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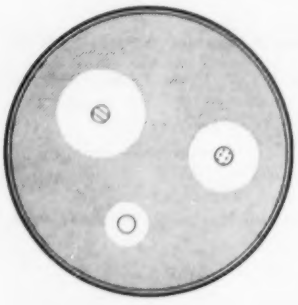

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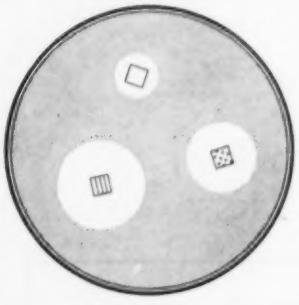

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VOL. XVII OCTOBER, 1956 No. 65

The Role of the Ram in Vibriosis of Sheep

B. D. FIREHAMMER, M.S.; H. MARSH, D.V.M.; E. A. TUNNICLIFF, D.V.M., M.S.

Bozeman, Montana

ALTHOUGH vibriosis of sheep was first described in 1913 [1] and has been studied for many years both in this country and abroad, the manner in which the ewe is infected is not known. It is generally accepted that infection in cattle is transmitted from the bull to the cow during breeding.[2-5] The only report indicating transmission by the ram is that of Buxton[6] who presented circumstantial evidence in 1929.

Field investigations of natural outbreaks and experimental procedures have been carried out by the Montana Veterinary Research Laboratory since 1930 in an attempt to determine the role of the ram in the disease. Groups of ewes were inoculated at the time of breeding, bred to artificially inoculated rams, or inseminated with suspensions of semen and virulent *Vibrio fetus* organisms. It was felt that exposure to pathogenic strains of *V. fetus* at the time of breeding would simulate natural infection, if the rams were the infective source. Additional groups of ewes were inoculated at other periods of the sexual cycle to determine the susceptibility to infection before breeding and at various stages of pregnancy.

NATURAL OUTBREAKS

The histories of two natural outbreaks in experiment station flocks are presented because in each instance the breeding records of the rams and the ewes were available.

Montana Experiment Station.—In 1942, vibriosis occurred in the Montana Experiment Station flock of 2,000 ewes, resulting in over 300 abortions. The abortion distribution and breeding records for 1,345 of the ewes which were divided into 25 breeding lots were obtained. Twenty-nine rams were used, 14 singly (table 1) and the remaining 15 in five groups of 3 rams each (table 2). Six of the rams which were used singly bred two lots of ewes each.

The 14 rams that were used individually bred 745 ewes that were in 20 lots, ranging in number from 6 to 62 ewes per lot. Breeding of nine of the lots started on

TABLE 1—Abortion Distribution in Lots of Ewes Bred with Individual Rams (1942)

Ram No.	Lot No.	Breeding started	No. of ewes	Abortions	Abortions (%)
1129	1	Oct. 27	17	6	35.3
1129	2	Nov. 15	37	0	0
K194	3	Oct. 27	61	13	21.3
K194	4	Nov. 15	44	1	2.2
5631	5	Oct. 27	36	5	13.8
5631	6	Nov. 15	43	3	6.9
1140	7	Oct. 27	17	2	11.7
1140	8	Nov. 15	35	0	0
F2010	9	Oct. 27	25	5	20.0
F2010	10	Nov. 15	52	1	1.9
C6	11	Nov. 12	6	0	0
C6	12	Nov. 15	40	1	2.5
2606	13	Oct. 27	62	11	17.7
1549	14	Oct. 27	58	10	17.2
K3134	15	Oct. 27	30	6	20.0
K3104	16	Oct. 27	54	9	16.6
529	17	Nov. 12	27	2	7.4
K1002	18	Nov. 12	9	0	0
1476	19	Nov. 15	41	0	0
FLJE	20	Nov. 15	51	2	3.9
Total			745	77	

The abortion rate of ewes in aborting lots was 12.4 per cent; in ewes bred October 27, 18.5 per cent; and in ewes bred November 12 or 15, 2.6 per cent.

Contribution from the Montana Veterinary Research Laboratory (Montana Experiment Station and Livestock Sanitary Board cooperating), Agricultural Experiment Station, Montana State College, Bozeman. Paper No. 377 journal series.

This work was supported in part by a grant from American Cyanamid Co., Lederle Laboratories Division, Pearl River, N. Y.

These experiments are phases of a regional attack on the sheep vibriosis problem in which research agencies in Colorado, Idaho, Montana, Utah, and Wyoming are participating.

The authors express their appreciation to Mrs. Alice Fluegel who assisted with the bacteriological studies.

October 27; breeding of the remaining 11 lots started on either November 12 or 15. A total of 77 ewes aborted in the 15 lots that suffered losses. The incidence of abortion ranged from 1.9 to 35.3 per cent, with

TABLE 2—Abortion Distribution in Lots of Ewes Bred with 3 Rams per Lot (1942)

Breed of rams	Lot No.	Breeding started	No. of ewes	Abortions	Abortions (%)
Hampshire	21	Nov. 15	121	7	5.7
Corriedale	22	Nov. 15	120	8	6.6
Romney	23	Nov. 15	120	9	7.5
Suffolk	24	Nov. 15	120	8	6.6
Columbia	25	Nov. 15	119	5	4.2
Total			600	37	

The abortion rate was 6.16 per cent.

a total morbidity of 12.4 per cent for the 617 ewes in the aborting lots. Although five lots were abortion-free, only 2 rams (K1002 and 1476), each of which bred only one lot of ewes, did not have abortions in at least one lot of ewes bred.

Abortions in the nine lots of ewes which were placed with the rams on October 27 ranged from 11.7 per cent to 35.3 per cent, with an incidence of 18.5 per cent for the total of 360 ewes (table 1). In comparison, the incidence of abortion in the 11 lots of ewes which were placed with the rams on November 12 or 15 ranged from no abortions (5 lots) to 7.4 per cent, with an abortion rate of 2.6 per cent for the total of 385 ewes. The reason for this significant difference in abortion rates between the lots bred on the two different dates is not known.

The possibility that rams used at the earlier breeding date were infected while those used later were not, is apparently eliminated by the fact that 5 of the rams were used on both dates. For all 5 of these rams, the ewes bred in the first lots had higher losses due to abortion than the ewes bred in the second lots. In two instances (rams 1129 and 1140), abortions occurred in the first lot of ewes that were bred, but not in the second. It seems unlikely that the rams were the source of infection in these two instances.

The 600 ewes that were bred by groups of rams were placed in three lots of 120, one of 119, and one of 121 ewes (table 2). Three rams were put in with each lot of ewes on November 15. Each group of rams represented a different breed of sheep. Abortion losses occurred in all five lots and varied from 4.2 to 7.5 per cent, the average being 6.16 per cent.

United States Sheep Experiment Station.—In 1952, Nordby [7] reported abortion in 668 of 2,513 ewes at the U. S. Sheep Experiment Station at Dubois, Idaho. A number of the fetuses were brought to the Montana Veterinary Research Laboratory where cultures yielded *V. fetus.*

The ewes were separated into 116 breeding lots consisting of 9 to 53 ewes, each with its own breeding ram. Abortions or stillbirths occurred in 112 of the 116 lots. The abortion rate in the 112 lots varied from 4.3 to 65.2 per cent. The 668 abortions represented 26.2 per cent of the 2,457 ewes in the aborting lots.

Although cultures were not made from fetuses from each of the aborting lots, it is probably safe to assume that few, if any, of the abortions were due to causes other than vibriosis. If the ewes were infected by the rams during breeding, it would follow that nearly all of the rams used were carrying *V. fetus,* which seems unlikely.

Wiggins [8] reviewed the lamb production records of 1,768 of the U. S. Sheep Experiment Station ewes which were in the flock in both 1952 and 1953 and of 692 young replacement ewes which were added in the fall of 1952 after the vibriosis outbreak. Twenty-one of the rams which were used in the fall prior to the outbreak were used again in the fall of 1952. The percentage of ewes having immature lambs in 1953 was slightly below the average for the period of 1940 to 1951, both in the older ewes and in the younger replacement ewes. Fetuses that were cultured at the Montana Veterinary Research Laboratory in the spring of 1953 showed no evidence of *V. fetus* infection. The failure to find evidence of infection the following season is typical of the observations made during other investigations of vibriosis in sheep. Although vibriosis may recur in subsequent seasons, this is not commonly the case.

EXPERIMENTAL PROCEDURE

MATERIAL AND METHODS

Source and Management of Sheep.—All sheep used were obtained from flocks which had no clinical history of vibriosis and were negative to a blood serum-agglutination test before use. Inoculated sheep were maintained in isolation for the duration of the experiment.

Cultures Used.—All cultures used were ovine

strains of *V. fetus* isolated from aborted fetuses at the Montana Experiment Station. Some were cultures that had been lyophilized shortly after isolation and were so stored until a short time before use. Other cultures were isolated from aborted fetuses and maintained on artificial mediums for a short time prior to use. All cultures produced abortion in pregnant guinea pigs following intracardiac injection of saline suspensions of organisms in pathogenicity trials just before experimental use. In some instances, additional pathogenicity trials were made by the intravenous inoculation of pregnant ewes.

Inoculation of Rams.—Four rams that were later used to breed ewes were inoculated with cultures that had been recovered from aborting guinea pigs. Rams H–4021 and L–4000 were inoculated intravenously, ram H–4024 directly into one testicle, and ram L–5040 via the epididymis and vas deferens.

Inoculation of Ewes via the Uterine Os.—A total of 42 ewes were inoculated at breeding time, in four experiments, by injecting suspensions of *V. fetus* either just within or against the uterine os. Seven of the ewes were bred immediately after inoculation, while 34 were bred first and then inoculated. Ewes in heat were selected from the flock by the use of aproned teaser rams. As these experiments were carried out in four different years, there was some slight variation in technique. In the first three trials, totaling 37 ewes, the growth on a 48-hour, 16-mm. agar slant was suspended in 2 to 3 ml. of saline solution and used to inoculate each ewe. In the last experiment, each ewe received half of the growth on a 48-hour, 16-mm. slant, suspended in 1 ml. of saline.

The uterine os was visualized by the use of a 1- by 8-inch glass speculum. Inoculation was made using a 5-ml. syringe equipped with a 12-inch needle with the end filed smooth. When possible, the tip of the needle was inserted just within the os; when this could not be done, the material was expelled against the os.

Ten ewes were inoculated two heat periods prior to breeding, using a similar technique. Half of the growth of a 48-hour, 16-mm. agar slant, suspended in saline, was used as the inoculum for each ewe.

Insemination with Infected Semen.—Sixteen ewes were inseminated with semen mixed with saline suspensions of *V. fetus*. The culture used (4532) was one that had been lyophilized six days after the initial isolation was made from an aborted fetus. The culture produced abortion in pregnant guinea pigs in pathogenicity trials made before and during the time the insemination work was being carried out. A culture from the same lyophilization run produced abortion in a ewe that was inoculated intravenously late in pregnancy, although this culture had been carried through ten transfers on artificial mediums after recovery from lyophilization.

The growth on 48-hour agar slants was suspended in physiological saline and the turbidity adjusted photoelectrically to a value between tubes 3 and 4 on the McFarland scale. Bacterial suspensions were made daily and were mixed with an equal quantity of semen just before use. Plate counts made on semen-Vibrio suspensions that were allowed to stand overnight at room temperature did not indicate any bactericidal effect by the semen. Semen was collected daily from a donor ram, using a water-jacketed artificial vagina. Microscopic evaluation of the semen was made to insure its quality.

Ewes in heat were identified by a vasectomized ram equipped with a marking harness. Marked ewes were placed in an elevated squeeze and the uterine os was visualized through a glass speculum. The semen-Vibrio suspension (0.4 ml.) was injected just within the os, using a 2.0-ml. glass syringe fitted with a 0.2-ml. serological pipette. With the volume of fluid employed, there was usually some return flow.

After insemination of the lot of ewes was completed, a breeding ram was left in the pen to breed any of the ewes which had not conceived as a result of the insemination. Four of the ewes

TABLE 3—Record of Ewes Inoculated Two Heat Periods Before Breeding

Ewe No.	Ram No.	Times rebred	Inoculation route	Lambing record	Culture results*	
					Uterus	Placenta
T–3	T–9853	1	Intravenous	None	....	....
W–108	T–9853	....	Intravenous	Normal	—	....
Z–4	T–9853	....	Intravenous	Normal	—	....
Z–30	T–9853	2	Intravenous	None	....	....
U–99	T–9853	....	Uterine os	Normal	—	—
Z–106	T–9853	2	Anterior vagina	None	....	....
Z–161	T–9853	....	Uterine os	Normal	—	—
Z–96	T–9853	....	Anterior vagina	Normal	—	—
W–52	T–9853	....	Uterine os	Normal	—	—
Z–107	T–9853	....	Anterior vagina	None	....	....
Z–91	C–8219	1	Uterine os	Normal	—	—
Z–123	C–8219	....	Uterine os	Normal	—	—
T–111†	T–9853	....	Uterine os	(Ewe died in third month)		
T–1	T–9853	....	Uterine os	Normal	—	—

* Results of examination for *Vibrio fetus* at time of lambing; other organisms were not identified.

† Ewe T–111 died from unknown cause. Cultures from the ewe and twin fetuses in the uterus were negative for Vibrio.

returned and were bred by the ram. None of the ewes was reinoculated.

Intravenous Inoculation of Pregnant Ewes.—Thirty ewes were inoculated intravenously at periods ranging from one to four months after breeding. Of the 30 ewes, 18 received half of the organisms on a 48-hour, 16-mm. agar slant, 1 received half of the growth on a 24-hour slant, 5 received the 48-hour growth from one fifth to three agar slants of 20-mm. width, and 6 were inoculated with 1 ml. of a saline suspension equivalent to McFarland tube No. 5. Four different strains of *V. fetus* were used.

Culture Studies Following Birth or Abortion.—Cultures were made at necropsy from all lambs that were born dead or died within the first few days after birth. In the later experiments, uterine swabs made after lambing or abortion were cultured, as were the fetal membranes when they were obtained in satisfactory condition. Cultures were routinely made on slants and semisolid mediums (0.15 % agar), containing 10 per cent sterile horse serum, made up from a basal medium previously described.[5] Plate cultures were made using Difco blood-agar base and 5 per cent defibrinated rabbit blood. All cultures were incubated under an atmosphere of 15 per cent CO_2, although work under way at the present time indicates that partial evacuation of the air is equally effective.

Inoculation Prior to Breeding

Fourteen ewes were inoculated while in heat and were bred at the second following heat period (approximately 34 days after inoculation). Of the 14 ewes, 4 were inoculated intravenously and 10 via either the uterine os or the anterior vagina (table 3). The inoculum, irrespective of the inoculation route, consisted of half of the 48-hour growth from a 16-mm. agar slant, suspended in saline. Strain 4849–X, which had just been reisolated from an inoculated guinea pig that aborted, was used to inoculate all of the ewes.

Nine of the ewes lambed normally, 4 were not pregnant (dry), and 1 died from unknown cause in the third month of pregnancy. Cultures from the ewe which died, as well as from the fetuses in her uterus, were negative for Vibrio. Uterine cultures, which proved to be negative for Vibrio, were made from the ewes when they lambed. The fetal membranes from 7 of the ewes were obtained for culture and found negative for *V. fetus*.

Two of the dry ewes were inoculated intravenously and two via the anterior vagina. There is a possibility that the high proportion of dry ewes was due to the low fertility of the aged ram T–9853, rather than as a result of the inoculation with *V. fetus*. Unfortunately, a semen evaluation was not made until the fall following use, when his semen was found to be of low quality.

A month after inoculation, the 4 ewes inoculated intravenously showed the following blood-agglutinin titers: W–108, 1:100; Z-30, 1:50; and Z–4 and T–3, both 1:25. Two months after inoculation, the titer of T–3 was up to 1:100 but, at three months, none of the ewes showed a significant titer. The ewes inoculated via the vagina or uterine os did not show any response to the inoculation.

TABLE 4—Record of Inoculation of Ewes at Breeding Time

Trial No.	No. of ewes	Inoculation route	Lambed	Did not lamb	Other
1	3	Uterine os	1	2*	0
2	10	Uterine os	9	1	1†
3	14	Uterine os	12	2	0
4	15	Uterine os	9	6	0
4	4	Intravenous	4	0	0
Total	46		35	11	1

* Ewe 461 was killed four months after inoculation; she was not pregnant. Uterine cultures were negative for *Vibrio fetus*.

† Ewe L–89 died three days after she returned to heat and had been inoculated for the second time. *Vibrio fetus* was recovered from the horns of the uterus.

Inoculation of Ewes at Time of Breeding

Viable suspensions of *V. fetus* were injected intravenously into 4 ewes and into, or against, the uterine os of 42 others at the time of breeding (table 4). Since the inoculation of these animals was carried out in four different experiments, they will be considered separately.

Trial 1.—Three ewes were inoculated by injecting suspensions of *V. fetus* into the uterine os, just before breeding, while the ewes were in heat. Two of the ewes (317 and 461) had previously received similar injections during the diestrous period. Strain 693, which was used to inoculate the third ewe (461), had been carried on artificial mediums for eight months, but retained its virulence as shown by the abortion of 7 ewes which, in another experiment, received intravenous injections of the culture. Strain 782, which was used to inoculate ewes 317 and 351, was recovered ten days prior to use from an aborted fetus.

Ewe 317 failed to lamb, and there was no evidence of early abortion. Ewe 461 was killed four months after inoculation and found to be nonpregnant. Cultures from her uterus were negative for Vibrio. Ewe 351 lambed normally and, although smears from a uterine swab revealed Vibrio which resembled *V. fetus*, cultures were negative.

Trial 2.—Ten ewes were inoculated by injecting suspensions of *V. fetus* against the uterine os immediately after breeding. All cultures used were derived from strain 2052, which had been isolated from a fetus nine months previously and carried in the lyophilized state for six months.

Of the 10 ewes, 5 were inoculated with a culture that was recovered from an aborting guinea pig (culture 2173), and 4 with a culture recovered from the liver of a guinea pig which died but did not abort. In each case, the inoculum consisted of the growth on a 16-mm. agar slant suspended in saline. Five ewes developed low blood titers which were not complete at any dilution.

One ewe (L–89) was inoculated with a culture recovered from the epididymis of an inoculated ram. This ewe did not conceive as a result of the original breeding and returned to heat 17 days later, at which time she was rebred and inoculated with a suspension of strain 2094. Three days after the second inoculation, the ewe was found dead, probably because of bloat. Cultures from the horn of the uterus yielded *V. fetus.*

Of the remaining 9 ewes, 8 lambed normally and 1 was not pregnant. Cultures made at lambing time from vaginal swabs and from the cotyledons were negative for Vibrio. The dry ewe was carried through two successive pregnancies without evidence of infection.

Trial 3.—Ten ewes were inoculated via the uterine os soon after copulation and 4 just before copulation. Culture 2209, which was recovered from the uterus of ewe L–89 at necropsy, was used for all inoculations. The culture was held in the lyophilized state for six months and then passed through 2 pregnant guinea pigs, producing abortion in each case, prior to inoculation of the ewes. The inoculum for each ewe consisted of the growth on a 16-mm. agar slant, suspended in 3 ml. of saline.

Of the 14 ewes, 12 lambed normally and 2 were dry. One ewe (M–39), subsequently found to be dry, showed a blood titer of 1:50 three and a half months after inoculation. At five months, the titer had dropped to partial agglutination at 1:25. The ewe was killed at this time; no evidence of pregnancy was found and cultures from the uterus were negative for *V. fetus.* This was the only ewe that showed more than a trace of agglutination at any time during the experiment. The 13 remaining ewes were carried through an additional pregnancy without evidence of infection.

Trial 4.—Fifteen ewes were inoculated via the uterine os and 4 intravenously, with suspensions of *V. fetus,* strain 4849, immediately after copulation. Each ewe, regardless of the route of inoculation, received a saline suspension of one half of the organisms from a 48-hour, 16-mm. agar slant. This strain was one which had been lyophilized after isolation from an aborted lamb. Abortions followed its use in pregnant guinea pigs in pathogenicity trials just prior to this experiment. In another experiment in which the same strain was used, the intravenous inoculation of ewes in the third or fourth month of pregnancy resulted in abortion in 6 of 7 ewes.

Of the 15 ewes inoculated via the uterine os, 9 lambed normally while 6 were dry. There was no evidence of abortion and uterine cultures made from the ewes that lambed were negative for *V. fetus.* All 6 of the dry ewes were bred the following fall and lambed normally without evidence of infection.

One of the 15 ewes (U–108) inoculated via the uterine os showed a titer of 1:800 one month after inoculation. This titer was maintained through the fourth month of pregnancy, but fell to 1:200 in the fifth month. She was killed one week after giving birth to normal twin lambs. Cultures from the heart blood, liver, spleen, kidneys, udder, and uterus were negative for *V. fetus.* This was the only ewe inoculated via the uterine os which showed a titer of any significance.

The 4 ewes which received intravenous inoculations lambed normally. One month after inoculation 2 of the ewes showed blood titers of 1:50. One ewe maintained this titer to the fourth month of pregnancy, while the other showed only a trace of agglutination after the first month. None of the ewes showed complete agglutination at any dilution five months after inoculation. All 4 ewes were carried through an additional pregnancy without evidence of infection.

Breeding with Inoculated Rams

Intravenously Inoculated Rams.—Ram H–4021 was inoculated intravenously with a suspension of strain 2102 and ram L–4000 with a suspension of strain 2209. Both strains were passed through pregnant guinea pigs, producing abortion just before inoculation of the rams. Ram H–4021 was placed with 10 ewes 22 days after inoculation.

All of the 10 ewes lambed normally and none showed agglutinins in the blood. The ram was killed three months after inoculation and cultures from his blood and genital organs were negative for *V. fetus.* Nine days after inoculation the ram showed a titer of 1:800, which fell to 1:50 by the time of necropsy.

Ram L–4000 started breeding a lot of 10 ewes nine days after inoculation. During the time that the ewes were being bred,

semen was collected twice and cultured for *V. fetus* with negative results. All of the ewes lambed normally although 2 developed titers of 1:25. The ram showed a titer of 1:200 one week after inoculation but, at three months, the titer had dropped to partial agglutination at 1:50. The ram died from other causes 11 months after inoculation and cultures from the genital organs were negative for *V. fetus*.

Rams Inoculated via the Genitals.—Ten ewes were bred to ram L–5040, nine days after a suspension of strain 2209 was injected into the epididymis and vas deferrens. The ram developed a blood titer of 1:1,600 by the time breeding started. All of the 10 ewes lambed normally and none developed titers. The ram was used in the two following breeding seasons without evidence of having transmitted vibriosis. Twenty-eight months after inoculation, the animal was killed and cultures of the genitalia were found negative for *V. fetus*. The blood titer at that time was 1:200.

Ram H–4024 received an intratesticular injection of strain 2102, 22 days before he was placed with a lot of 10 ewes. Of the 10 ewes, 7 lambed normally while 3 were dry. None of the ewes developed agglutinin titers. The ram was killed three months after inoculation. *Vibrio fetus* was isolated from the right epididymis, which was enlarged to approximately four times the normal size. This was the only 1 of the 4 inoculated rams from which *V. fetus* was recovered.

INSEMINATION WITH INFECTED SEMEN

Sixteen ewes were inseminated with semen to which viable suspensions of *V. fetus*, strain 4532, had been added.

Of the 16, 11 (68.75 %) conceived as a result of the single insemination and lambed without evidence of vibriosis; 1 ewe (X–107) was dry; and 4 returned to heat and were rebred but not reinoculated. All 4 lambed normally.

Uterine cultures made after lambing from 14 of the ewes were found negative for *V. fetus*. The remaining ewe which lambed was not cultured. The fetal membranes from 8 of the ewes were cultured and were also found negative bacteriologically.

TABLE 5—Ewes Inoculated Intravenously at Different Stages of Pregnancy

Strain	Month inoculated	Ewe No.	Inoculum*	Result	Abortion, days after inoculation
4849	1	Z–128	1/2 slant	Lambed	....
	1	Z–80	1/2 slant	Lambed	....
	1	Z–35	1/2 slant	Lambed	....
	1	Z–28	1/2 slant	Lambed	....
	2	Z–83	1/2 slant	Aborted	2
	2	Z–11	1/2 slant	Aborted	5
	2	Z–68	1/2 slant	Aborted	17
	2	Z–166	1/2 slant	Aborted	3
	3	Z–62	1/2 slant	Aborted	5
	3	Z–170	1/2 slant	Lambed	....
	3	W–99	1/2 slant	Aborted	24
4532	1	W–13	1 ml.†	Dry	....
	1	Y–18	1 ml.†	Lambed	....
	1	X–40	1 ml.†	Lambed	....
	1	X–120	1 ml.†	Lambed	....
	1	X–12	1 ml.†	Lambed	....
	4	Z–114	1 ml.†	Aborted	17
225	2	413	1 slant	Aborted	24
	2	339	1/5 slant	Lambed	....
	3	353	3 slants	Died, 24 hr.	....
	3	356	1 slant	Aborted	34
	3	372	1 slant	Aborted	41
693	3	413	1 slant	Aborted	60
	3	339	1 slant	Aborted	39
	3	501	1 slant	Aborted	41
	3	3000R	1 slant	Aborted	27
	3	453	1 slant	Aborted	60
	3	457	1 slant	Aborted	5
	3	1879	1 slant	Aborted	32
2209	3	J–16	1 slant	Aborted	60

* Cultures were grown on 16-mm. agar slants, with the exception of strain 225 which was grown on 20-mm. slants. Forty-eight hour cultures were used, with the exception of strain 2209 which was made from a 24-hour growth. Suspensions in physiological saline were made shortly before use.

† The growth on agar slants was suspended in saline and adjusted photoelectrically to a turbidity equivalent to McFarland tube No. 5.

Three ewes, including the dry ewe (X–107) and 2 which were rebred (U–85 and U–122), showed low blood titers. Ewe X–107 showed a titer of 1:25 14 days after she was inoculated but lost the titer by the end of the first month. Ewe U–85 showed a titer of 1:25, 21 days after inoculation, but, at 56 days, it had dropped to partial agglutination at 1:25. Her titer remained at this level until lambing time, when it rose to 1:50.

The third ewe, U–122, did not show a titer until lambing time, when she developed a titer of 1:50. It is possible that the low titers developed by these animals indicated infection and possibly some interference with conception, as 1 was dry and the other 2 were rebred. The rise in titer at lambing time, although cultural evidence of infection could not be found, may have been due to a physiological response associated with parturition.

Intravenous Inoculation of Pregnant Ewes

Thirty pregnant ewes were inoculated intravenously with a saline suspension of *V. fetus* at various stages of pregnancy (table 5). Of the 30 ewes, 11 were placed in three groups which were inoculated with strain 4849, one, two, and three months, respectively, after breeding.

The 4 ewes inoculated when one month pregnant lambed normally, while 6 of the 7 ewes inoculated when either two or three months pregnant aborted. Ewe Z–170, inoculated when three months pregnant, lambed normally. Uterine cultures at lambing time from ewes which lambed normally were negative for Vibrio. Abortions occurred from two to 24 days after inoculation in the aborting ewes. Vaginal swabs positive for *V. fetus* on culture were not obtained from any of the ewes more than five days after abortion. One ewe died on the fifth day after she aborted, possibly due to a secondary infection. Two ewes (Z–68 and Z–166) were killed five months after they aborted. Cultures from the heart blood, gallbladder, liver, spleen, kidneys, udder, ovaries, uterus, cervix, and vagina of both animals were negative for Vibrio. The remaining 8 ewes were carried through an additional pregnancy without evidence of infection.

A second attempt was made to produce abortion in ewes at the end of the first month of pregnancy. Five ewes were inoculated intravenously with suspensions of strain 4532 one month after they were bred. Of the 5 ewes, 4 lambed normally and 1 was dry. Uterine and placental cultures made at lambing time did not reveal evidence of infection. A sixth ewe (Z–114), inoculated with the same strain while in the fourth month of pregnancy, aborted 17 days after inoculation. *Vibrio fetus* was isolated from the fetal membranes and from the twin fetuses.

A total of 13 ewes were inoculated either during the second or the third month of pregnancy, using *V. fetus* strains 225, 693, and 2209. Of the 13 ewes, 11 aborted from vibriosis at periods of five to 60 days after inoculation; 3 of them aborted 60 days after inoculation while only 3 aborted in less than 30 days after inoculation. One ewe (353) died 24 hours after inoculation, while another (339) lambed normally. The ewe which died was inoculated with the growth from three 20-mm. agar slants, a much heavier inoculum than was used on any of the other ewes. The ewe that lambed received one fifth of the growth from a 20-mm. agar slant, the smallest amount of inoculum used.

Discussion

The investigation of vibriosis at the Montana Experiment Station in 1942 revealed that 12 of 14 lots of ewes developed vibriosis after a different ram was used to breed each lot (table 1). Consequently, if the rams were the source of ewe infection, 12 of them were infected with *V. fetus*. Although this could conceivably occur, it was considered unlikely. Two of the rams which each bred a second lot of ewes had abortions in the first lot but not in the second. If the rams were infected, it would seem unlikely that the second lots bred would escape infection. The rate of abortions in the nine lots of ewes bred on October 27 was 18.5 per cent, while it was only 2.6 per cent for the 11 lots bred on November 12 or 15. The reason for this significant difference in rate of infection is not known.

In the same epizootic, abortions occurred in each of five lots of ewes that were bred by separate groups of rams, although each group represented a different breed of sheep.

The 1952 vibriosis epizootic at the U. S. Sheep Experiment Station indicated that if the rams were the infective source, it would be necessary that 112 of the 116

rams used were carrying the organism at breeding time. Again, this proportion of infected rams was considered improbable.

The 14 ewes which were inoculated with *V. fetus* two heat periods prior to breeding, either via the uterine os or intravenously, did not abort. However, 4 were not pregnant, 2 from each group, possibly due to the low fertility of the ram used, or as a result of the exposure to the organism. Of the 4 ewes inoculated intravenously, 3 developed blood titers of 1:50 or higher following inoculation, but the titers disappeared by the third month.

Although none of the 46 ewes which were inoculated at the time of breeding aborted, the number of dry ewes (11) was unusually high. This was particularly so in trial 4, where 6 of 15 ewes inoculated via the uterine os did not lamb. Although none of these dry ewes showed blood titers, it is possible that the organism established itself long enough to cause a sterility problem.

Infertility is an important aspect of the syndrome of bovine vibriosis but, as yet, the investigation of natural outbreaks has not revealed a similar situation in sheep. Losses in sheep are due to abortions, which usually occur in the fifth month of pregnancy, or to weak or dead full-term lambs. The high proportion of dry ewes in some of the inoculated groups could be due to a situation similar to that found in bovine vibriosis. Sterility in sheep due to vibriosis may be greater than is suspected, or it may be that, in nature, ewes become infected during pregnancy rather than at breeding time resulting in a problem of abortion rather than one of infertility.

The failure to transmit vibriosis by breeding to rams that were experimentally inoculated is of doubtful significance due to the fact that the organism was definitely established in only 1 ram. *Vibrio fetus* was reisolated from the epididymis of this ram three months after inoculation, but there was no proof that the organism was being eliminated in the semen during the breeding time.

The failure to produce vibriosis by insemination with suspensions of semen and *V. fetus* confirmed the results from the four experiments in which ewes were inoculated at the time of breeding. It seems difficult, if not impossible, to produce abortion by inoculation at breeding time. Field investigations and experimental work indicate that the ram is not the principal source of ewe infection.

The intravenous inoculation experiments revealed that although abortions can be produced quite readily by inoculation in the second, third, or fourth month of pregnancy, ewes are quite resistant to infection during the first month (table 5). This is in agreement with the results obtained by Lindenstruth *et al.*[10] who found that ewes early in pregnancy were not as readily infected by feeding cultures or by intraperitoneal injections as ewes late in pregnancy. Lee and Scrivner[11] produced abortion in 4 of 5 ewes fed cultures in the fourth month of pregnancy, while 3 ewes which were fed cultures in the second or third month lambed normally. Although McFadyean and Stockman[1] apparently established infection early in pregnancy, it would appear that it can be done only with difficulty.

The failure of aborting ewes, as well as inoculated ewes which were dry, to show evidence of infection the following season is in agreement with the findings of Marsh *et al.*[12] The observation that at least the majority of infected ewes lose the infection before the next lambing season is also substantiated by the failure to isolate *V. fetus* from the vaginas of aborting ewes for more than a short period following abortion and by the results of cultures made at necropsy of 2 ewes five months after abortion.

Unpublished work carried out at the Utah Experiment Station[13] and the Montana Veterinary Research Laboratory has shown that rams may harbor Vibrio sp. in the preputial mucus that are similar in morphology to *V. fetus*. These organisms are H_2S-positive and catalase-negative, while *V. fetus* strains are H_2S-negative and catalase-positive. Preliminary work indicates that these organisms are not involved in vibrionic abortion and are probably similar to the organisms isolated from the reproductive tracts of cows and bulls by Bryner and Frank.[14]

SUMMARY AND CONCLUSIONS

1) Abortions occurred in the majority of the lots of ewes in two natural epizootics of vibriosis in flocks that were divided into small breeding lots. If the rams were the source of the infection, the ma-

jority of them were infected, which was considered unlikely.

2) Fourteen ewes were inoculated, 4 intravenously and 10 via the uterine os, during the second heat period prior to breeding. Although none of the ewes aborted, 4 did not have lambs, possibly as a result of temporary infection.

3) At breeding time, 4 ewes were inoculated intravenously and 42 via the uterine os. Although none of the 46 ewes aborted, 11 did not lamb, possibly due to sterility resulting from a temporary infection. Eight of the 11 were carried through another pregnancy without evidence of infection.

4) The investigation of naturally occurring vibriosis has not indicated a sterility problem such as is found in bovine vibriosis, but rather one of abortion late in pregnancy. However, the high proportion of nonpregnant ewes in some of the experimentally inoculated groups may have been due to transitory infection.

5) The insemination of ewes with semen containing pathogenic *Vibrio fetus* organisms failed to result in abortion.

6) The intravenous inoculation of ewes in different stages of pregnancy showed that ewes are resistant to infection during the first month of pregnancy, but are quite readily infected during the second, third, or fourth month.

7) Ewes which neither aborted nor lambed following inoculation with *V. fetus*, as well as ewes that aborted following inoculation, were carried through an additional pregnancy without evidence of infection.

8) Both field and experimental findings, indicate that the ram is not the source of ewe infection. The relative ease with which abortion can be produced by inoculation late in pregnancy indicates the possibility of natural infection during gestation from infected premises or some other as yet undetermined source.

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An Important Stage in the Development of the Os Cornu (Bone Core) of the Horns of the Sheep

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THE MOST important and interesting feature in the development of the os cornu in the sheep is the formation of bone in the mesenchymal analage over the area of the horn site on the frontal bone of the skull. In the initial, unusual step, cartilage appears in the superficial stratum of the intramembranous frontal bone.

This phase in the primordium of the os cornu has been remarked upon by several workers in the development of hollow horns of the ox, sheep, and goat.[2, 9, 14, 16, 17, 19] The work was mainly of microscopic character and a large number of sheep heads were required for sectioning. The material used was 50 fetuses, from a few weeks to full development, and 40 sheep heads varying in age from a few hours to 5 years.

The tissue was fixed in 5 per cent formaldehyde, decalcified by either Carleton's method [4] or Clayden's method,[5] embedded in paraffin, sectioned, and stained with haematoxylin and eosin or safranine and thionine.

Histological examination of successive ages of fetuses indicates that a thickening of the frontal bone in the horn site can be recognised in the male at 90 to 100 days' development while in the female it is not recognised until the first or second week after birth. This thickening of the frontal bone in the horn site is the primordial frontal pedicle. The bone spicules in this thickened region are horizontally arranged and are parallel to the outer surface of the frontal bone. As the male fetus advances in age, the frontal pedicle becomes thicker and at the same time the most superficial bone spicules, which are close to the periosteum, change gradually from the horizontal to the vertical direction (fig. 1.). At 110 to 120 days, the first sign of the future horn core can be recognized as cartilage cells formed on the superficial layer of the frontal bone which until now is of intramembranous development (fig. 2 and 2a). The cartilage formation is seen only in the elevated part of the frontal bone which later will be the frontal pedicle. This peculiarity of the development of the os cornu, the occurrence of intramembranous and endochondral ossification forming at the same time in the frontal bone, was also recognised in the antler of the deer as stated by Macewen [12] and Noback.[15] Macewen said "From the germinal layer the osteoblasts may evolve into bone directly, or pass through an intermediary cartilaginous stage, from which they become finally converted into bone. These two methods of direct and indirect transference of osteoblasts into bone may be seen in the same antler at the same time, though usually at some distance apart." This important point can be explained by first summarizing the two methods of bone formation and then comparing them with the development of the os cornu in the sheep.

The development of membranous bone as stated by Jordan and Kindred,[10] Cajal,[18] Arey,[1] Maximow and Bloom,[13] Trautmann and Fiebiger,[20] and Ham [7] begins when a cluster of mesenchymal cells differentiate into osteoblasts. After the osteoblasts appear, it is not long before some of them secrete or deposit the bone matrix in the form of bone spicules. Some of the osteoblasts surround themselves with the intercellular substances and become differentiated into osteocytes or bone cells while the others proliferate and arrange them-

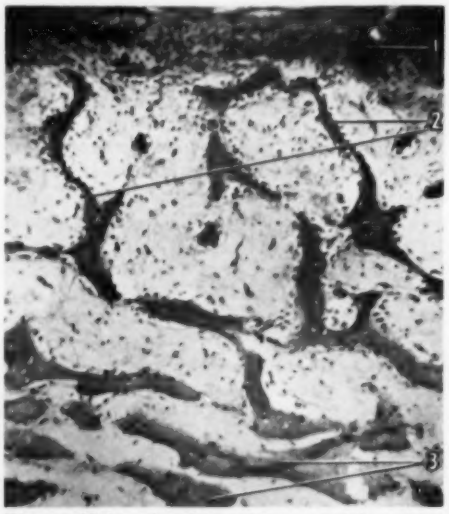

Fig. 1—Male ovine fetus, 120- to 130-day stage. The uppermost bone spicules of the frontal bone at the horn site beginning to attain a vertical direction to the outer surface of the frontal bone. 1—Periosteum; 2—bone spicule vertically placed; 3—bone spicule horizontally placed.

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selves in an epithelioid layer upon the surface of the bone spicules already formed. This process goes on continually until the bone is fully developed. For a long time the membranous bone has a spongy character. It consists of irregular plates and bars which branch and unite with one another. The spaces between the bone spicules are filled with embryonic connective tissue rich in blood vessels and dividing cells. This tissue is gradually transformed into myeloid tissue with osteoclasts. The connective tissue surrounding the growing bone gives rise to the periosteum. As the bone spicules increase in size and thickness, the spaces between them are correspondingly narrowed. Thus, the character of the spongy bone will change from a structure consisting of large spaces with little bone to one of narrow spaces with much bone. When bone becomes the predominant feature of the tissue, it is said to be compact or dense bone. In this manner are developed the dense deep or inner and the superficial or outer compact plates of the frontal bone, with spongy bone joining the two.

The development of cartilage begins when the mesenchymal cells (connective tissue cells) become rounded, lose their cytoplasmic processes and are closely packed together. Each cell forms a thin capsule around itself; the blended capsules compose the first matrix or intercellular substance, which is laid down in increasing amounts between the cells, and gradually pushes them apart. The mesenchyme surrounding the cartilage primordium forms a special layer, the perichondrium, which merges gradually with the cartilage on one side and the adjacent embryonic connective tissue on the other.

This microscopic picture of the osseous tissue of the frontal bone in the region of the primordial os cornu shows the first sign of cartilage forma-

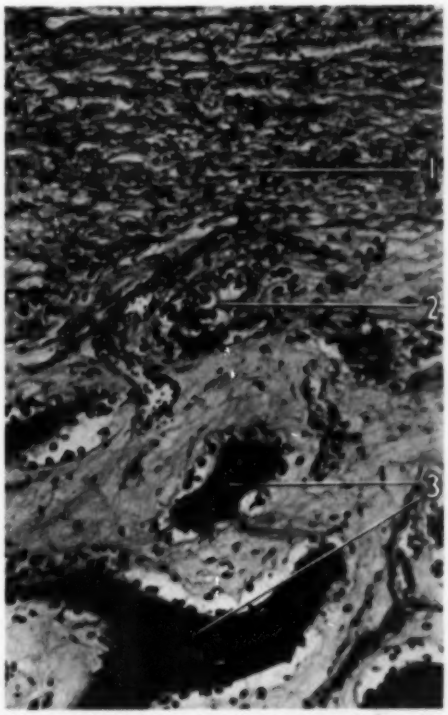

Fig. 2—First stage of cartilage formation at the horn site of a 110- to 120-day male ovine fetus. x 145.
1—Perichondrium; 2—cartilage cells; 3—bone spicules.

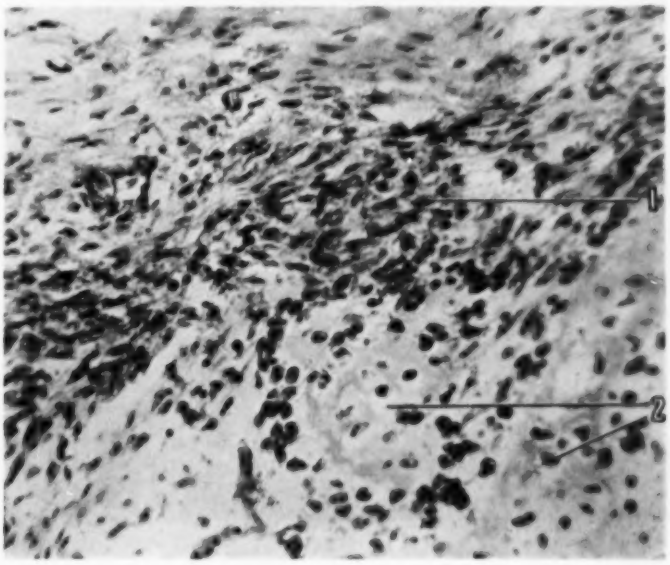

Fig. 2a—Early stage of cartilage formation at the horn site of a 130- to 140-day male ovine fetus.
1—Perichondrium; 2—cartilage cells.

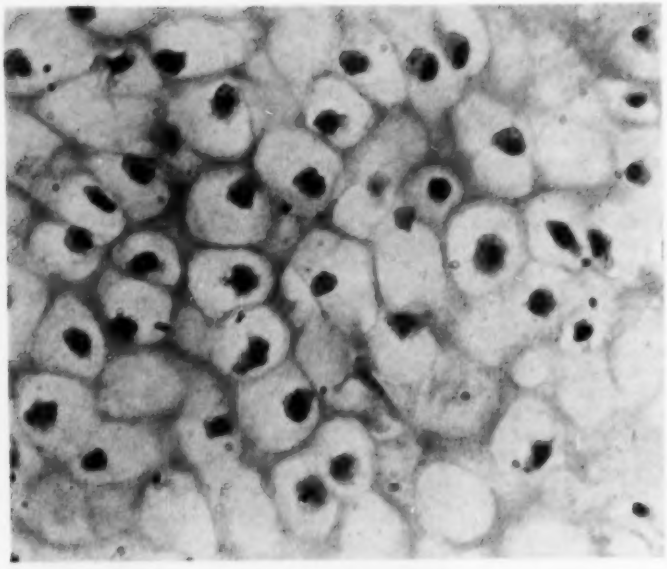

Fig. 3—Cartilage cells of a 140- to 150-day male ovine fetus. x 520.

Fig. 4—Cartilage formation (primordial os cornu) of a 140- to 150-day male ovine fetus. x 110.
1—Dermis; 2—perichondrium; 3—cartilage cells.

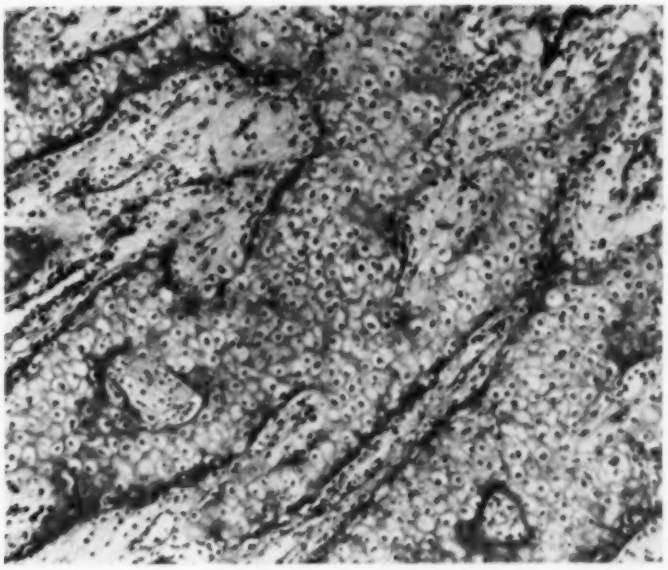

Fig. 4a—Cartilage trabeculae (os cornu) of a first- to second-week male ovine fetus. x 100.

tion. The cells of the deeper mesenchymal layer, covering the upper surface of the previously formed bone spicules, come close together and lose their protoplasmic processes, become rounded, and secrete a thin capsule about themselves. These cells begin to form the intercellular substance of the cartilage which is clearly demonstrated (fig. 2 and 2a). The cartilage formation is the first sign of the primordial os cornu which will develop from a separate cartilaginous center laid on the osseous tissue of the frontal process.

During the rest of the gestation period of the male fetus, *i.e.*, between the age of 120 to 145 days, the cartilage cells are formed in increasing

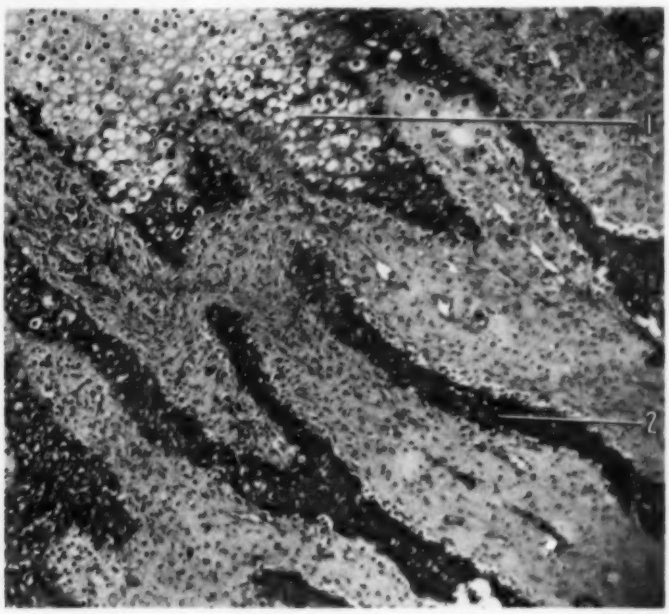

Fig. 5—Apex of the os cornu of a 4- to 6-week male ovine fetus. x 260. 1—Cartilage trabeculae; 2—bone spicules.

Fig. 6—Cartilage of the apex of the os cornu at an advanced stage of replacement by bone (8- to 12-week male ovine fetus). x 220.

numbers at the apex of the cone-shaped process of the frontal bone, and lie close to the perichondrium on one side, and to the previously formed bone spicules on the other. The cartilage cells are more or less spherical, are surrounded by a thick capsule, and have a shrunken nongranular cytoplasm (fig. 3).

Because of the activity of the perichondrium during the first few weeks after the birth of the lamb, the cartilage cells are formed in increasing numbers, and are closely gathered into large groups forming cartilage trabeculae, which are seen all along the superficial surface of the cone-like process of the frontal bone (fig. 4 and 4a). The cartilage trabeculae are arranged in a position vertical to the free surface. Close to the perichondrium, the cartilage cells are more or less flattened, while deeper down they are more rounded. The ground substance is scarce in the newly formed cartilage masses or trabeculae, while in the deeper or previously formed ones it appears as a solid mass in which separate cartilage cells are scattered at some distance from one another. The perichondrium surrounding the cartilage sends down cords of mesenchymal cells to become connective tissue between the cartilage strands. The superficial layers of the perichondrium consist of densely arranged collagenous connective tissue, while its deeper layers show some cartilaginous characteristics, forming chondroblasts and chondrocytes.

The growth of cartilage takes place in two different ways as stated by Ramon-Cajal,[18] Maximow and Bloom,[12] Trautmann and Fiebiger,[20] and Ham.[9] The first is interstitially, in which the mature cartilage cells divide and give rise to new cartilage cells. The latter cells can in turn give rise to new cartilage cells and to more intercellular substance. The second is by appositional growth, in which new layers of cartilage are apposed to one of its surfaces. This depends on the activity of the perichondrium which keeps transforming layers of connective tissue into cartilage. From examining the successive stages of cartilage formation in sections, the writer came to the conclusion that the growth of cartilage in the horn of sheep is through the appositional mechanism. It is clear in the successive ages of 140 to 150 days of prenatal life and during the first 2 to 6 weeks of the postnatal life of the sheep that the perichondrium is very active. It continues to add new cartilage cells as mentioned before. At the same time, bone tissue begins to replace the older cartilage tissue.

This replacement of cartilage by bone is recognized for the first time at this stage and be-

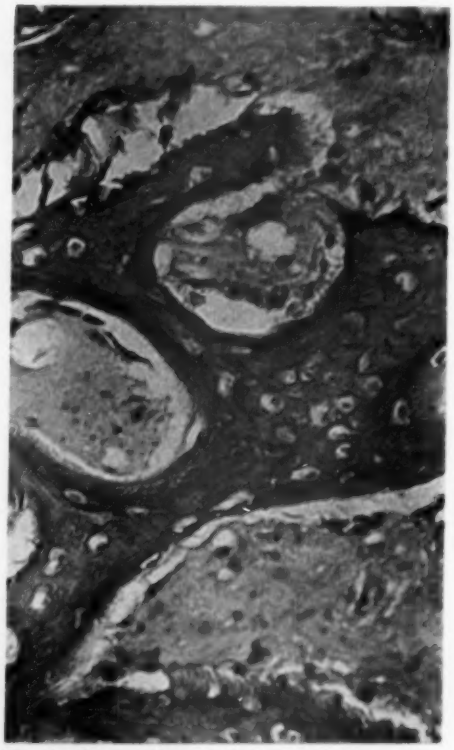

Fig. 7—Osseous tissue at the apex of the os cornu of a 16-week female ovine fetus. x 260.

comes clearer as the lamb reaches the age of 6 weeks. At this age, the osseous tissue of the growing os cornu presents two stages of development of cartilage. The outer zone, which is close to the perichondrium, is still forming new cartilage cells, especially in the region of the apex (fig. 5). The activity of the perichondrium in forming cartilage cells is not as great as in the previous stages and, moreover, decreases as it is traced towards the base of the os cornu. In the middle or central zone of the os cornu, the previously formed cartilage is being replaced by bone in the usual manner as stated by Ramon-Cajal,[18] Maximow and Bloom,[13] Trautmann and Fiebiger,[20] and Ham.[9]

In female horned sheep, the first sign of cartilage formation in the horn site can be recognised in lambs of 2 to 3 weeks of age. The process of cartilage formation and its growth resembles that described in male lambs.

At the age of 12 weeks, in both sexes, no more cartilage is formed and the previously formed cartilage is gradually replaced by bone (fig. 6 and 7).

There are different views on the method of development of the bone core in hollow horned animals; a review of the literature reveals three different theories.

The first theory is supported by Youatt,[21] Lataste,[11] Durst,[6] Zietzschmann,[22] and Brandt.[3] These authors state that the bone core in cavicorn animals develops through intramembranous ossification with no separate center from the frontal bone; it is not an independent bone but only a part of the frontal bone. Durst supported this theory by explaining that the horn core begins with a thickening in the preosseous substance in the horn site, that the thickening of the bone is due to the rapid proliferation of the preosseous tissue of the outer stratum of the frontal bone, and that intramembranous ossification occurs when this preosseous tissue grows into lamellae on the external layer of the frontal bone. From this, Durst concluded that the bone core is not an individual bone but is only a part of the frontal bone.

The second theory is that the bone core originates by intramembranous ossification but with an independent center of ossification separate from that of the frontal bone. This was the idea of Fambach,[7] who stated that the frontal pedicle is first formed in the horn site, then the osteoblasts in the horn site penetrate the periosteum, and the subcutaneous connective tissue forms an isolated bone nucleus, which is the os cornu, and that the supraperiosteal activity continues until the remaining periosteal stripes have become ossified.

The third theory, which agrees with the writer's findings, assumes the development of the bone core from an independent cartilaginous center, which soon becomes indistinguishably connected with the pedicle of the frontal bone. This theory was supported by Sandifort,[19] Numan,[16] Nitsche,[14] Gadow,[8] Atzkern,[2] and Ottolenghi.[17] Their statements refer to hollow-horned animals, which include mainly the ox, sheep and goat.

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Physiological Studies of the Vagal Nerve Supply to the Bovine Stomach
III. Procaine Effects on the Dorsal Vagal Trunk

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THE TECHNIQUE of applying electrodes to the vagus nerve previously described [1] appeared to lend itself to further study of the eructation mechanism.[2] For this purpose, two sets of electrodes were applied to the dorsal vagal nerve trunks of normal adult cattle as described below. Each electrode assembly consisted of an empty polyethylene tube in addition to, and accompanying, the insulated wires. This extra tube was used for the purpose of injecting procaine hydrochloride solution to produce a nerve block anterior or posterior to the point of electric stimulation.

MATERIALS AND METHODS

Thoracotomies were performed on 5 adult grade dairy cows and on 1 grade Hereford steer for the placement of double electrode assemblies on the dorsal vagal trunk. Three of these 6 animals were used for the studies here reported. One death occurred during anesthesia. (Chloral hydrate was used intravenously in 5% solution; death was from respiratory failure.) One animal was discarded because of injuries suffered while coming out of general anesthesia; a third animal (steer) was sent to slaughter when it was found that the electrode assemblies had not remained in place around the nerve trunk.

The animals used in these experiments were surgically prepared by a series of two operations: the first to prepare a rumen fistula, and the second a thoracotomy to apply the electrodes to the thoracic vagus. The procedure of the thoracotomy was essentially the same as that previously described,[1] except that two sets of electrodes were applied to the dorsal vagal nerve trunk; one set was approximately 10 cm. anterior to the diaphragm, the other set approximately 10 cm. anterior to the first. These electrode assemblies each consisted of three 18-gauge silver wires* enclosed in polyethylene tubing.** Methyl cellulose† was injected into the area between the wire and the tubing to prevent possible ascending infection. The three electrode wires then were braided and the empty tubing intertwined with these. The free ends of the wires and the tube were brought out through the thoracic wall and, after injecting 2 ml. of crystalline penicillin (200,000 units) into the empty tubing and sealing its end with a flame, the assembly was buried in the subcutis. In the last animal operated upon, the tubing from one electrode was entwined with the wires of the other electrode, to obviate a second incision at the time of administering procaine.

The 3 animals were allowed a recovery period of two to three weeks, and then were used for experimental purposes at approximately weekly intervals. Their usefulness, for experiments requiring vagal stimulation, extended to five and a half months for 1 animal; the other 2 are still being used after two and a half and one and a half months, respectively. The duration of usefulness for experiments requiring two functional sets of electrodes on the dorsal vagal trunk, however, appeared relatively short (4 to 6 weeks). This will be discussed at length below.

On the day of a given experiment, an animal was prepared for the recording of pressure changes in the anterior and posterior dorsal sacs of the rumen, reticulum, and cardia.[2] Nitrogen was insufflated into the rumen through a sealed Dougherty rumen plug at a constant rate of flow, selected to induce regular eructation without a rise in intraruminal pressure. Using aseptic technique and local anesthesia, the free ends of the wires and the tubes were isolated. After testing for wire breakage with an ohmmeter, two of the wires were connected to the terminals of an electronic square-wave stimulator. A microphone was placed over the esophagus just posterior to the pharynx, and the amplified events in the esophagus were recorded independently, using a signal marker with a remote control switch (graphs 1 and 2, bottom line).

The general experimental procedure then consisted of recording pressure changes and base line pressure before, during, and following the administration of 4 per cent procaine hydrochloride so-

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* American Platinum Works, Newark, N. J., No. 0.999 fine, half-hard.

** P.E. 200, Clay-Adams, New York, N. Y.

† Rower Dental Mfg. Corp., Boston, Mass.

TABLE 1—Summary of Observations on Events in the Rumen of Dairy Cows Before and After Administration of Procaine to the Dorsal Vagal Trunk

Animal (No.)	Date	Procedure	Effect on eructation rate/minute			Effect on rumen contraction rate/minute			Effect on rumen base line pressure (mm. Hg)		
			Before*	During**	Change	Before*	During**	Change	Before*	After**	dP/dT†
846	11-4-55	Procaine block of dorsal vagal nerve trunk	1.0	1.0	0.0	2.0	3.5	+1.5	3	20	+0.03
846	11-11-55	Procaine block of dorsal vagal nerve trunk	0.8	0.4	−0.4	2.2	1.2	−1.0	8	17	+0.01
846	11-19-55	Procaine block of dorsal vagal nerve trunk	2.0	1.6	−0.4	2.0	2.2	+0.2	0	8	+0.01
1004	12-29-55	Procaine block of dorsal vagal nerve trunk	1.1	0.4	−0.7	1.8	3.0	+1.2	0	15	+0.2
1004	1-11-56	Procaine block of dorsal vagal nerve trunk	1.0	1.7	+0.7	1.6	2.3	+0.7	0	30	+0.03
1362	2-11-56	Procaine block of dorsal vagal nerve trunk	1.2	2.0	+0.8	1.0	2.2	+1.2	0	30	+0.01
1362	2-18-56	Procaine block of dorsal vagal nerve trunk	0.7	2.7	+2.0	0.4	1.3	+0.9	0	45	+0.01
846	11-11-55	Stimulation alone	0.8	2.0	+1.2	2.2	5.3	+3.1	0	0	0.0
	11-11-55	Procaine (anterior), stimulation (posterior)	0.4	0.6	+0.2	1.2	1.7	+0.5	17	17	0.0
1004	1-11-56	Stimulation alone	1.0	5.6	+4.6	1.6	3.3	+1.7	0	0	0.0
	1-11-56	Procaine (anterior), stimulation (posterior)	1.7	2.0	+0.3	2.3	2.4	+0.1	30	25	−0.03
1362	2-18-56	Stimulation alone	0.7	3.0	+2.3	0.4	0.7	+0.3	0	0	0.0
	2-18-56	Procaine (anterior), stimulation (posterior)	2.7	8.6	+5.9	1.3	1.0	−0.3	45	5	−0.07
1362	2-11-56	Stimulation alone	1.2	5.0	+3.8	1.0	1.0	0.0	0	0	0.0
	2-11-56	Procaine (posterior), stimulation (anterior)	2.0	11.0	+9.0	2.2	2.0	−0.2	20	0	−0.13

* Values obtained just prior to treatment (procaine and or stimulation). Those values represent averages computed from events occurring over a five-minute period.

** Values obtained during the effect produced by procaine or during the effect produced by stimulation following procaine. The values on procaine effects represent events occurring over a five-minute period, starting ten to 15 minutes after procaine administration. The values computed during stimulation represent events occurring during intervals of stimulation varying from two to ten minutes.

† Rate of change in rumen base line pressure (mm. Hg/second); dP = change in pressure, mm. Hg; dT = change in time, seconds.

lution on to the dorsal vagal trunk. In the trials involving study of the effects of nerve stimulation, the pretreatment recordings consisted of both nonstimulated and stimulated periods. The procaine solution was then injected on to the nerve via the electrode not being used for stimulation. After recording the effect of procaine application, the nerve again was subjected to the same eructation stimulus. The stimulus was continued until the rumen presure returned to zero, or until it became apparent that little or no effect was being obtained.

Due to the time and expense involved in surgically preparing and subsequently maintaining these animals, it was sometimes necessary to use the animals for other experiments on the same day these trials were conducted. However, in those cases, the animals were allowed to completely recover normal ruminal activity before performing these trials.

The stimulus selected was that which, in the individual concerned, would increase the eructation rate and, if possible, the rate of rumen contraction, with a minimum of applied voltage. Stimuli having a frequency of 10 per second, a pulse duration of 0.5 millisecond, and voltage ranging from 40 to 100 volts were used in the present series. It appeared that intermittent stimulation of two to three seconds' duration, followed by pauses of the same duration, was more effective than continuous stimulation over the same total time.

At the completion of an experiment, the electrode wires and the tubing for procaine injection were cut short. Then 5 ml. of crystalline penicillin (500,000 units) was placed in the tubes and they were fused shut at the terminus with a flame. These were then replaced subcutaneously, penicillin-streptomycin ointment placed in the surgical wound, and the incision closed with two No. 2 chromic catgut sutures.

Results

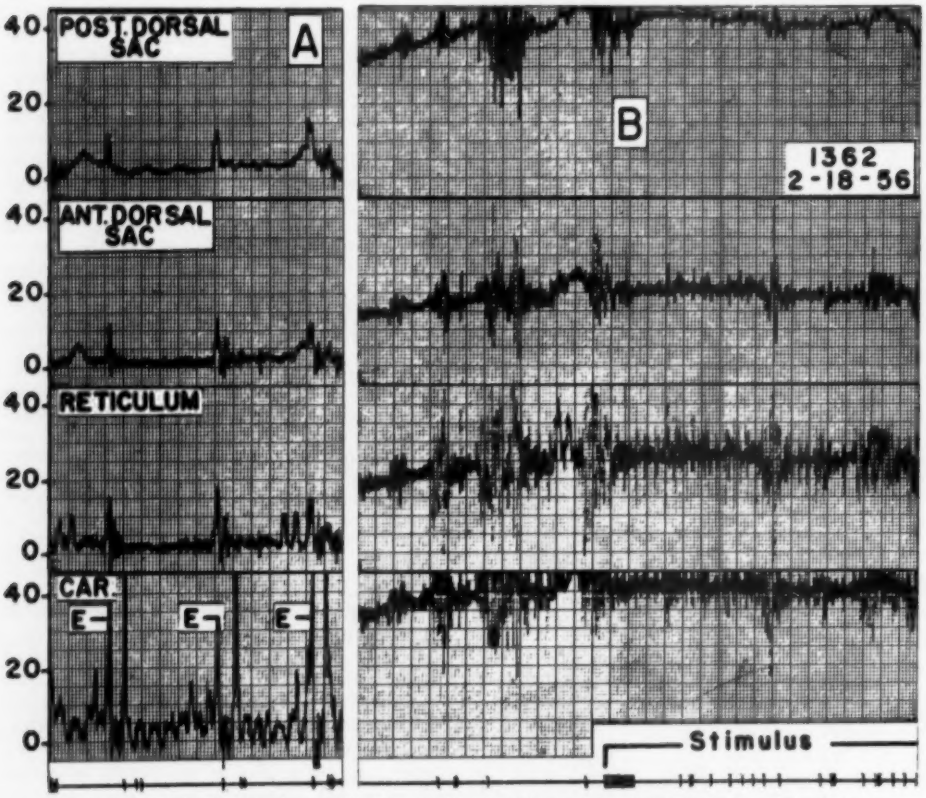
In seven trials of the 3 animals (table 1), the application of procaine to the dorsal vagal nerve trunk in all cases resulted in some degree of decreased eructation efficiency with an increase of 8 to 45 mm. Hg in base line pressure in the posterior dorsal rumen sac. The effect of procaine on eructation, in so far as the single criterion of rate (as distinguished from over-all efficiency of eructation) was concerned, was variable, consisting of an increase in three trials, a decrease in three trials, and no change in one trial. However, the animals showing an increased eructation rate attained the highest intraruminal pressures. The increased intraruminal pressure was also accompanied by an increase in rumen rate in all but one trial, in which

a relatively small increase (9 mm. Hg) in rumen pressure occurred (No. 846, table 1).

Stimulation of the dorsal vagal nerve trunk previous to treatment with procaine was associated with a definite increase in the rate of eructation in all four trials, and an increase in rate of rumen contraction in three of four trials.

Following the administration of procaine, stimulation was attempted posterior to the point of nerve block in three trials and anterior to the point of nerve block in one trial. The animals bloated to varying degrees in all four trials (table 1) with visible and palpable distension of the flank. The effect of stimulation seemed dependent upon the point of stimulation relative to the point of procaine block.

Stimulation of the nerve trunk posterior to the point of procaine application was accompanied by slight increases in the rate of eructation in 2 animals and a marked increase in the third. The rumen base line



Graph 1—Effects of procaine application to the dorsal vagal trunk anterior to point of electric stimulation in an unanesthetized adult Guernsey cow.

Simultaneous recordings of pressure changes (reading from top to bottom) in posterior dorsal rumen sac (gas pocket), anterior dorsal rumen sac (floor), reticulum (floor), and antrum of the cardia.

Record A shows spontaneous motor events, prior to procaine application. Eructations (marked E) are occurring on both primary and secondary rumen contractions.

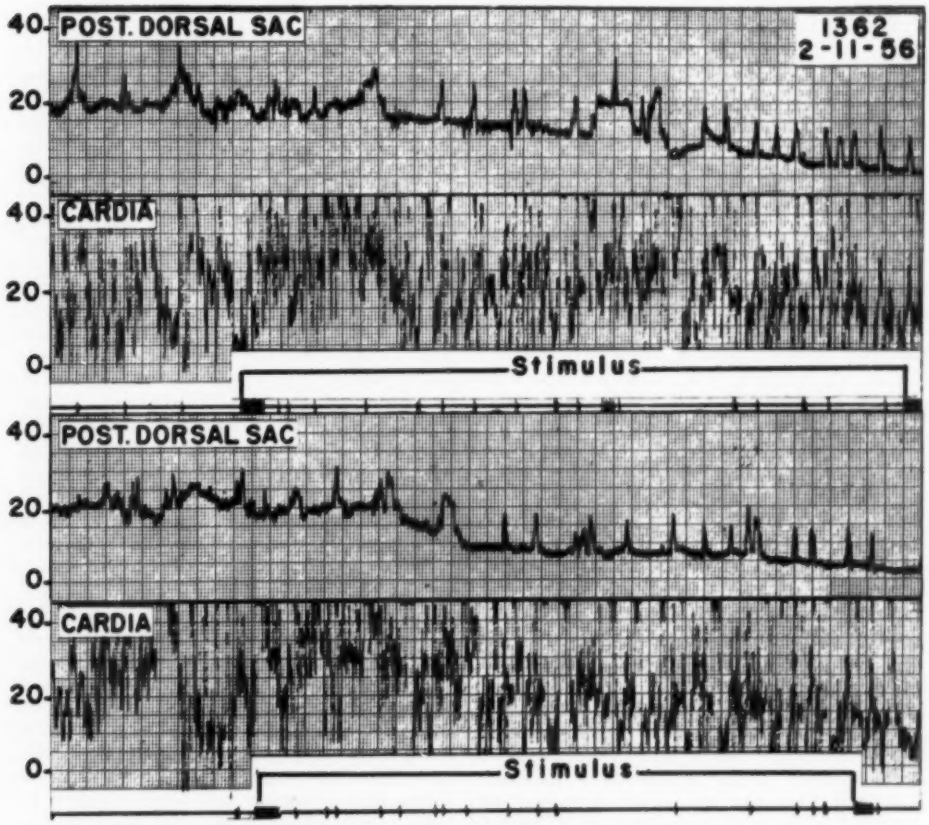
Record B was taken 20 minutes after injection of 4 per cent procaine into the anterior electrode assembly; notice bloating.

At stimulus, the dorsal vagal trunk was stimulated through posterior electrode assembly. An eructation response is seen (signal marker, bottom line), but eructations appear inefficient, since rumen base line pressure comes down slowly. Nitrogen insufflation rate, records A and B, was 5 liters per minute.

Stimulus used: 10 cycles per second, 0.5 millisecond pulse duration, 100 volts. Horizontal axis, 1 large division = 5 seconds; vertical axis, 1 large division = 5 mm. of mercury pressure. Signal marker (bottom line): 1 mark = eructation; 2 marks = swallow; long mark = start of stimulus.

pressure remained elevated in animal 846 and decreased slowly upon long, continued stimulation in the other 2 animals tested (1004, 1362). The animal showing the most rapid fall in pressure per unit of time (1362) required nine and a half minutes of stimulation to drop from 45 to 5 mm. Hg (fig. 1). It was also apparent (table 1) that this animal showed the largest increase in eructation rate during stimulation. Thus, eructation efficiency (rate x volume) would appear decreased by anterior block plus posterior stimulation. The rumen contraction rate showed only slight changes during these trials of stimulation posterior to the point of block.

Stimulation of the nerve anterior to the point of procaine block was accomplished in one experiment only (animal 1362, Feb. 11, 1956). Animals 846 and 1004 had suffered damage to their anterior electrodes by the time this anterior-stimulation, pos-



Graph 2—Effects of application of procaine to the dorsal vagal trunk posterior to point of electric stimulation in an unanesthetized adult Guernsey cow.

Upper two traces are simultaneous recordings of pressure changes (reading from top to bottom) in posterior dorsal rumen sac (gas pocket) and antrum of the cardia. Left-hand side of record (before stimulation) shows moderate bloat 20 minutes after injection of 4 per cent procaine hydrochloride into posterior electrode assembly. Stimulus applied over period indicated to anterior electrode assembly. During stimulation, there was rapid eructation rate and rather rapid return to normal base line pressure. Fifteen minutes after the stimulus was removed (lower two traces), the animal had become bloated again and was again brought to zero base line pressure by stimulation anterior to point of block. Nitrogen insufflation rate was 5 liters per minute.

Stimulus used: 10 cycles per second; 0.5 millisecond pulse duration, 50 volts. Horizontal axis, 1 large division = 5 seconds; vertical axis, 1 large division = 5 mm. of mercury pressure. Signal marker (bottom line): 1 mark = eructation; 2 marks = swallow; long mark = start of stimulus; long mark plus short mark = end of stimulus.

terior-block type of experiment was attempted. This procedure resulted not only in marked increase in the eructation rate but also relatively rapid return of the rumen pressure to the base line (fig. 2 and table 1). That is, over-all eructation efficiency upon stimulation anterior to the point of block resembled the preprocaine efficiency. Only a slight (negative) effect of this stimulus on rumen contraction rate was observed.

Once the rumen pressure began to rise (graph 1), many periods of restlessness were evident (rapid up-and-down strokes of the styluses). If the degree of overdamping was increased somewhat (by adjusting the drip clamp) greater clarity was achieved, as in the "posterior dorsal sac" trace (graph 2).

Attention may be called to two previous observations.[2] The first would be, judging from the record of events taking place in the reticulum and two compartments of the rumen (graph 1, A), that simple transmission of pressure changes is not the rule in this organ. Only those pressure changes occurring in the immediate vicinity of the opening of the recording tube are apparent on the tracing produced, with the exception of those accompanying eructations. Further study is being given to this point. Secondly, it has been regularly observed that eructations may occur during normal intraruminal pressures on either or both the primary and secondary ruminal contractions (graph 1, A). This appears to be the normal state of affairs in some of the animals studied, even without additional administration of gas.

DISCUSSION

The data do not permit a simple explanation, and the present paper is submitted as a report of progress. The number of animals is not great; preparation of animals with double electrodes was difficult and limiting. Yet, the direction of change, at least, seems clear. The dorsal vagal trunk appeared to be important in eructation of cows. Application of procaine to this trunk was accompanied by a tendency toward bloating, a situation deriving largely from decreased volume of gas removed per eructation, since the eructation rate was not markedly or regularly changed (table 1).

The afferent fibers of an eructation reflex may be more numerous than the efferent fibers in the dorsal vagal trunk, or may be more effectively stimulated artificially. In the former case, one possibility would be that the efferent fibers travel mainly in the ventral vagal trunk. Another possibility would be that efferent fibers may leave the dorsal trunk proximal to a point about 10 cm. anterior to the diaphragm. This last-mentioned possibility, if true, might indicate, as Dougherty [2] has suggested, that the efficiency of eructation is quite dependent upon events occurring in the esophagus itself.

In these experiments (table 1), the rate of rumen contraction appeared to increase somewhat upon the onset of bloating, a situation similar to that observed in field cases.[4] The rumen rate tended to increase during stimulation of the dorsal vagal trunk (before applying procaine, table 1). Duncan [5] found that in sheep the normal rhythmic activity of the rumen and reticulum was lost following total vagotomy.

A ruminoreticular motor center has been postulated in the sheep brain, by Iggo,[6] caudal to the intercollicular plane, and further studied by Clark.[7] Comline and Titchen [8] examined the reflex nature of esophageal groove contraction in calves.

The difficulties experienced in establishing, and more particularly in maintaining, these animals with two vagal electrode assemblies appeared to indicate that it would be best to use single electrode assemblies in subsequent experiments requiring access to the vagus, and to apply double electrode assemblies only in special cases.

Animals having implanted vagal electrodes are difficult to maintain, since their usefulness is dependent upon the absence of factors such as infection, wire breakage, or slipping of the electrode assemblies off the nerve. The preparation described presents three additional problems. They are: (1) Infectious organisms may gain access to the tube used for procaine injection by being injected with the procaine at the time of the next experiment; (2) the procaine tube appears more subject to breakage than the rest of the assembly; and (3) there seems to be a tendency to develop poor rumen motility after a time, with accompanying change in the consistency of the ingesta. Work is being done to determine possible causes of these last effects. As a part of this study, sections for histo-

logical examination were routinely taken from the vagus nerve anterior and posterior to the points of electrode attachment and also from the dorsal nucleus of the vagus at the time of slaughter.*

SUMMARY

1) Procaine hydrochloride solution was injected on to the dorsal vagal trunk during seven trials on 3 adult dairy animals, while recording the pressure changes in the rumen and the reticulum.

2) In all seven trials, eructation efficiency was impaired following procaine injection, with a rise in intraruminal pressure.

3) Electric stimulation of the nerve trunk anterior to, and posterior to, the point of application of procaine indicated that the dorsal vagal trunk may serve as an afferent pathway in an eructation reflex.

* This work was done with the cooperation of Dr. R. L. Kitchell of the Division of Veterinary Anatomy, University of Minnesota, St. Paul.

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Preliminary Report on Immunization of Animals Against *Micrococcus Pyogenes*

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MASTITIS caused by *Micrococcus pyogenes* is the most significant type of mammary disease in many dairy herds. In spite of optimistic reports of effective treatments for staphylococcic mastitis, no simple control measures have yet been developed. Histopathological observations of mammary glands chronically infected with *M. pyogenes* have demonstrated many granulomatous and suppurative foci not connected with the duct system. Treatments are not likely to be effective in such cases. In the authors' experience, cases of long standing resist treatment and remain as a reservoir of infection in the herd. The high prevalence of subclinical infections with *M. pyogenes* suggests some degree of natural or acquired resistance.

Farrell and Hitching[1, 2] found alum-precipitated toxoids better than alum tartrate toxoids or unmodified toxoids for antibody production. Richou *et al.*[3, 4] found antihemolytic titers of 140 international units (I.U.) following immunization of cows with a potassium alum-treated toxoid, whereas toxoid not treated with alum induced only 3 to 20 I.U. A staphylococcic toxoid was reported by Gould[5] and Wilson[6] to improve cases of staphylococcic mastitis when used therapeutically. Kearney and Maloney[7] and Collingson[8] found clinically apparent benefit from the use of staphylococcic vaccines; the former used an autogenous mixture of staphylococcic anaculture, whereas the latter injected a toxoid.

Ramon and associates[9–14] established that potassium alum and aluminum hydroxide as adjuvants enhanced the production of antihemolysins in cattle. They also noticed that cattle 2 years old and older developed the highest titers and that animals less than 6 months old developed a relatively low titer. Minett[15] found that alum-precipitated toxoid produced immunity against staphylococcic mastitis for at least three months.

Sheep are also susceptible to staphylococcic mastitis, and attempts have been made to immunize them against the infectious agent. McLay *et al.*[16] produced severe mastitis by intramammary injection of culture into ewes but found no benefit from treatment with staphylococcic toxoid. Richou and Holstein[3] produced an antihemolytic titer against staphylococci of 1 to 20 I.U. (average, 10 I.U.) in 5 ewes.

Parish and Clark[17] were able to demonstrate neutralizing antibodies in the serum of horses immunized with a toxoid when such serum was injected into mice, guinea pigs, or rabbits, prior to the injection of a lethal culture of staphylococci. Burkey[18] reported high precipitin titers and effective protection of rabbits against lethal doses of staphylococci when they had been previously injected intravenously or intradermally with a toxoid. Richou[19] found that a large dose (12 units) of toxoid was superior to 6 units for immunization of rabbits. Ramon and Richou[20] obtained high antihemolytic titers (600 I.U.) in horses with a staphylococcic toxoid-tapioca mixture. In other reports,[21, 22] they determined the duration of the immunity in rabbits against staphylococcic infection and demonstrated the beneficial effect of certain adjuvants. Ramon *et al.*[23] also reported that the production of *alpha* antitoxin was superior to the production of *beta* antitoxin by their vaccines. They also reported that inclusion of formalin-inactivated bacteria to their toxoid gave a higher and more persistent antibody titer.

Several methods of production of staphylococcic toxin and toxoid have been proposed. Bigger and Boland[24] suspended agar cultures in saline, centrifuged them, and dried the supernatant for storage. Burnet[25] found that traces of carbon dioxide above cultures enhanced the production of toxin. Slanetz *et al.*[26] found buffered tryptose agar was the best of several mediums for the production of potent toxins. He also reported that growth at 37 C. for 48 hours in an atmosphere of 20 to 25 per cent carbon dioxide gave optimum toxin production. Duthie and Wylie[27] obtained a large population of organisms and much hemolytic toxin by cultivation in Walbum's medium in an atmosphere of 80 per cent oxygen and 20 per cent carbon dioxide.

MATERIALS AND METHODS

The medium utilized for the production of Staphylococcus toxin was a modification of the medium of Walbum.[28] The ingredients were:

Fresh beef heart infusion (330.0 Gm. per liter)	1,000.0 cc.
KH_2PO_4	4.0 Gm.
$MgSo_4$	0.6 Gm.
Adjust to final pH 7.0.	

A fresh broth culture was inoculated in 3 per cent quantity into flasks of medium. Twenty per cent carbon dioxide and 80 per cent oxygen were introduced into the flask. It was sealed and incu-

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TABLE 1—The Effect in Rabbits of Experimental Formalin-Inactivated Vaccines Prepared from Highly Toxic Cultures of Micrococcus Pyogenes and Administered in Solid Pellet Form or Suspended in Mineral Oil or Aqueous Aluminum Hydroxide. The Immunity of the Rabbits Was Later Challenged by Subcutaneous Inoculation of a Toxic Culture of the Organism

Vaccine preparation	Dose (Gm.)	No. of rabbits	Anti-hemolysin titer (I.U.)	No. rabbits with local reaction after challenge				No. died	No. positive cultures at death or slaughter
				None	Slight	Moderate	Marked		
Pellet	0.1–0.5	5	3–6	0	0	5	0	0	None made
Pellet	1.0	5	13–25	1	1	3	0	0	None made
Mineral oil suspended	1.0	5	3–25	3	2	0	0	0	None made
Aluminum hydroxide	0.5 (1 dose)	5	2	0	0	0	5	2	5
Aluminum hydroxide	0.5 (3 doses)	15	1–32	5	9	1	0	0	0
*Commercial toxoid	1 ml. (3 doses)	10	4–32	3	7	0	0	0	0
None	0	10	0	1	1	2	6	4	6

* Commercial Staphylococcus toxoid: 1,000 dermonecrotic units per milliliter prior to detoxification.

bated at 37 C. for 48 hours in a rotating shaker. The culture was then centrifuged at 3,000 r.p.m. in an angle-head centrifuge for 30 minutes, and the supernatant was removed. The toxin was measured by titration with standard antitoxin according to the method of Hartley and Smith,[20] and the same method was used for measurement of antihemolysin levels.

Ten cultures of *M. pyogenes* isolated from aseptically collected milk of dairy cows were tested for ability to produce hemolytic toxin. One of these (strain 17 C) was selected because it was a high toxin-producer, and it was used in all subsequent work. Production of toxin seemed to be variable in different batches and many were discarded because of low hemolytic toxin level.

A whole broth culture containing a high concentration of toxin was used to prepare the immunizing agents. Formaldehyde was added to the culture to make a final concentration of 0.35 per cent. The mixture was held for three to 20 days at 37 C. The mixture was then vacuum-dried over a dehydrating agent at 5 C. or lower. This dried material was the basic ingredient for the vaccines. The first preparation was a pelleted vaccine made by mixing the dried detoxified culture with casein, egg albumen, or gelatin solutions. A 40 per cent solution of formolized gelatin was selected as the better pelleting agent. These pellets were implanted subcutaneously in rabbits. An anhydrous suspension in mineral oil of the powdered detoxified culture was also used for immunization of rabbits and 3 cattle. Another vaccine was prepared by suspending the dried detoxified culture in a washed 10 per cent aqueous suspension of aluminum hydroxide. This vaccine was injected into 20 rabbits, 6 cattle, and 6 sheep. A commercial Staphylococcus toxoid, which contained 1,000 dermonecrotic units per milliliter prior to formolization, was employed in three doses of 2 ml. each at a seven-day interval for immunization of 1 cow. Ten rabbits were injected with this material in a total amount of 1 ml. divided into three doses at a seven-day interval. At intervals after inoculation, the titer of antibodies was determined, and the rabbits were challenged by subcutaneous inoculation of a whole broth culture. The severity of the local reaction was recorded.

The cattle were vaccinated by intramuscular injection of one or three doses and were tested at intervals thereafter for the titer of antihemolysin. Four vaccinated lactating cows and 2 control unvaccinated cows were challenged by intramammary inoculation with 2 ml. of various dilutions of broth culture of strain 17 C, *M. pyogenes*.

Lactating sheep were vaccinated intramuscularly with the aluminum hydroxide vaccine and were tested for antihemolysin titer at weekly intervals thereafter. Two vaccinated and 1 control sheep were challenged by intramammary inoculation of a fresh broth culture three weeks after vaccination. Four vaccinated sheep and 1 control were challenged similarly nine weeks after vaccination. Cultures of strain 17 C, used for challenge of both cattle and sheep, contained from 74 million to 120 million organisms per milliliter before dilution, as determined by a surface plate count

TABLE 2—Results of Vaccination of Cattle with Micrococcus Pyogenes Antigens Suspended in Anhydrous Mineral Oil or Aqueous Aluminum Hydroxide

Animal No.	Vaccine used	Dose (Gm.)	Local reaction	Antihemolysin titer (I.U./ml.)		
				2 wk.	8 wk.	14 wk.
1	Oil	3.0	Severe	51	13	0.5
2	Oil	1.25	Severe	51	2	0.5
3	Oil	2.5	Severe	102	4	1.0
4	Al (OH)	2.0	Slight	102	(Challenged at 3 wk.)	
5	Al (OH)	1.0	None	100	32	8
6	Al (OH)	0.5	None	100	8	1
7	Al (OH)	2.0	None	100	16	8
8	Al (OH)	2.0	Slight	2	2	2
9	Al (OH)	2.0	Slight	64	16	8
11	Toxoid	4 ml.	None	...	(Challenged at 3 wk.)	

on Difco blood agar containing 6 per cent bovine blood.

RESULTS

Rabbits.—The results of trials in rabbits are summarized in table 1. The pellet vaccines produced marked local irritation and sterile abscess formation but gave considerable protection against subsequent challenge. Mineral oil suspensions conferred a high degree of immunity and were somewhat less irritating than the pellet vaccine. Aqueous suspension of vaccine in aluminum hydroxide usually evoked a high degree of immunity when administered in three doses at weekly intervals. The aluminum hydroxide vaccine produced little local reaction at the site of injection. These experiments in rabbits indicated that repeated doses of vaccine were much more effective than a single dose containing the same quantity of antigen. The commercial toxoid apparently gave as good protection as the experimental vaccines under the conditions of the experiment. Antihemolysin titers were variable and did not always indicate the resistance of the animal to challenge with virulent organisms.

Cattle.—The results of vaccination of 10 cattle are summarized in table 2. The vaccines suspended in mineral oil were irritating locally, but no definite abscesses developed. Antihemolysin titers were highest after three weeks and subsided gradually over a period of several months after vaccination. The aqueous vaccine suspended in aluminum hydroxide produced little or no local reaction and yet induced a high and persistent titer in all except 1 animal.

Four lactating, vaccinated cattle and 2 unvaccinated controls inoculated via the intramammary route developed a local inflammatory reaction in their mammary glands as described in table 3. The acute reaction in 16 quarters of vaccinated animals was less severe than that in the unvaccinated cows. Nine of 16 quarters in vaccinated animals became chronically infected. There were no signs of chronic mastitis in these other than a slight induration. One of the control animals was treated with antibiotics several times during the week subsequent to challenge because of impending gangrene, and she became permanently infected in spite of treatment. The other control animal was destroyed because of gangrene and impending death two days after inoculation.

The milk whey from cow 4 was found to have an antihemolysin titer of 12 to 51 I.U. after challenge, at the same time that the quarters were eliminating viable organisms. Milk from these quarters would not support growth of *M. pyogenes*. The blood from vaccinated cows was found to

TABLE 3—Effects of Immunization on Intramammary Challenge of Cattle with 2 ml. of Various Dilutions of Micrococcus Pyogenes*

Cow No.	Quarter	Time between vaccination and challenge	Dilution of challenge culture	Degree of acute reaction	Duration of infection
4	RF	21 da.	1:500	None	>29 wk.
	RR		1:100	None	>29 wk.
	LR		1:10	Slight	1 wk.
	LF		Undiluted	Slight	>29 wk.
8	RF	122 da.	1:500	Slight	1 wk.
	RR		1:100	Slight	1 wk.
	LR		1:10	Slight	>10 wk.
	LF		Undiluted	Moderate	2 wk.
9	RF	122 da.	1:500	None	≤ 1 wk.
	RR		1:100	None	≤ 1 wk.
	LR		1:10	Slight	>10 wk.
	LF		Undiluted	Slight	>10 wk.
10	LF	Not vaccinated	Undiluted	Marked	>10 wk.
11	RF	21 da.	1:500	None	> 3 wk.
	RR		1:100	None	> 3 wk.
	LR		1:10	Moderate	> 3 wk.
	LF		Undiluted	Moderate	1 wk.
12	RF	Not vaccinated	Undiluted	Marked	Died after
	RR		1:10	Marked	2 da.
	LR		1:100	Moderate	
	LF		1:500	Moderate	

*Animal 11 was given two doses 21 days apart of commercial toxoid, whereas the other animals were vaccinated with one dose aluminum hydroxide-absorbed toxoid-bacterin.

TABLE 4—Results of a Single-Dose Vaccination of Sheep with Aluminum Hydroxide Aqueous Vaccine and Subsequent Intramammary Inoculation of 1 ml. of Various Dilutions of a Toxic Culture of Micrococcus Pyogenes

Animal No.	Vaccine dose (Gm.)	Titer at 3 wk. (I.U.)	Time of challenge (wk.)	Dilution of challenge		Reaction to challenge
				Right	Left	
3	0.05	1.0	3	1:500	1:1,500	Slight
401	0.6	8.0	3	1:500	1:1,500	Slight
6	None	1.0	3	1:500	1:1,500	Slight
51	None	0.5	9	1:500	1:10	Moderate
28	0.05	0.5	9	1:500	1:10	Marked
27	0.15	4.0	9	1:500	1:10	Moderate
52	0.3	2.0	9	1:500	1:10	Moderate
412	0.6	2.0	9	1:500	1:10	Slight

inhibit *in vitro* growth of *M. pyogenes* in dilutions up to 1:20.

Sheep.—The results of vaccination of 6 sheep and their subsequent exposure to virulent cultures by the intramammary route are presented in table 4. There was no apparent local inflammatory reaction to vaccination, but antihemolysin titers were low in comparison with those in cattle. A chronic infection developed in many of the udders, and four of them became inflamed subsequent to the next parturition. There was no indication that the vaccine gave protection, but larger numbers are needed to provide definite results. On the basis of these trials, the proper dilution for intramammary challenge was probably about 1:10, using this strain of *M. pyogenes*.

Discussion

The results reported here agree with the findings of Ramon and associates that a mixture of staphylococcic toxoid and killed bacteria produces good antihemolytic titers. A large dose of antigen was found necessary for immunity in rabbits. Likewise, in cattle, the concentration and persistence of antibodies were roughly in proportion to the amount of vaccine used.

Poor results were obtained in immunization of sheep. Scrutiny of the experiment revealed that the batch of vaccine had been refrigerated in the dried form for four months prior to vaccination. During immunization, the sheep were lactating and were on poor pasture. These factors may have contributed to the low degree of protection obtained.

Higher titers of antihemolysins were attained after vaccination of cattle than have been observed in natural infection. The occurrence of antibacterial antibodies inhibitory to growth in milk and plasma is of considerable interest and indicates that vaccination should partially alleviate the severity of staphylococcic mastitis. Whole cultures inactivated and detoxified seem to offer more promise for immunization of cattle than do purified toxoids. The literature cited from French workers offers convincing evidence that a higher and more persistent level of antihemolysin can be stimulated by the whole cultures than by toxoid. Further trials in experimentally vaccinated cattle subsequently exposed to infection are necessary to establish the possible value of such vaccines in the field.

Summary

Vaccination of rabbits with preparations of *Micrococcus pyogenes* culture disclosed a protective effect from a combination of toxoid and formalin-killed organisms combined with an adjuvant, such as mineral oil or aluminum hydroxide. A commercial Staphylococcus toxoid had similar protective effects for rabbits.

Eight out of 9 cattle immunized with large doses of bacterin-toxoid developed high titers of antihemolysins that persisted at least 14 weeks. The severity of mastitis in 16 quarters of vaccinated cows challenged with viable toxic cultures was less than the reaction in five quarters of unvaccinated cows. A chronic infection developed, following challenge, in nine of the 16 quarters of vaccinated cows.

Immunization of sheep with bacterin-toxoid resulted in low titers and no evidence of protection against subsequent intramammary challenge.

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Submucosal Glands in the Bovine Colon

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DURING studies on the pathology of experimental bovine hyperkeratosis, a thickened area was observed in the colon mucosa. This was thought to be associated with the hyperkeratosis syndrome but was later found to occur in cattle not exposed to the etiological agent of hyperkeratosis. The present study was undertaken to clarify the occurrence and nature of this thickened area in relation to the normal and pathological anatomy of the bovine colon.

LITERATURE

Sisson and Grossman[22] mentioned the occurrence of Peyer's patches "in the beginning of the cecum and one in the first part of the colon" of cattle. Elias[6] reported the presence of glands in the submucosa of the ileum of the ox, "especially within the lymphatic patches of Peyer." Ramsey[20] described a "focal, chronic, cystic colitis about 10 to 20 cm. distal to the ileocecal valve" in a mucosal disease of cattle. Maurer *et al.*[16] mentioned a cecocolic lymphoid area as a frequent site of damage in rinderpest. Dunne *et al.*[5] described caseous nodules under the mucosal surface of the rectum and colon of pigs recovered from niacin deficiency. Histological examination revealed "invaginations of the mucosa into the submucosa, forming cyst-like cavities surrounded by lymphoid tissue." Biswal *et al.*[3] described grossly visible areas in the porcine colon which were found to be submucosal glands imbedded for the most part in lymphoid tissue. Klein,[14] quoted by Biswal, observed such submucosal glands in the colon of swine in 1878. Follis[8] also mentioned the normal occurrence of gland prolongations into the submucosal lymphoid tissue in the colon of swine.

Schultze[21] described the occurrence of submucosal crypts in relation to lymphoid tissue in apes and man. Further, he found these glands in ape embryos but not in human embryos. Schultze also credited Retterer with the first description of these structures in the colon of the guinea pig in 1892; however, Klein appeared to have antedated Retterer. Tomarkin[24] in 1893, Küchenmeister[15] in 1895, and Stöhr[23] in 1898 confirmed Retterer's findings. Stöhr stated that similar structures were observed by Retterer in sheep, cattle, and horses. Klaatsch[13] was quoted by Schultze as observing these glands in the anteater, while Orth[19] described submucosal glands in normal and diseased human colons. Brites[4] discussed the frequent occurrence of Lieberkühn's glands penetrating the submucosa of the sigmoid flexure in 24 human beings over 60 years of age. Such penetrations were not seen in younger individuals. Bargen[1,2] studied what were considered to be precancerous changes in the colon of man. Certain of these changes were interpreted as being similar to the appearance described by Schultze[21] and generally known as the "Schultze picture."[12]

MATERIALS AND METHODS

Colons from 52 cattle were collected at necropsy, or from a local slaughter house for gross and microscopic examination. The specimens were from animals ranging, approximately, from 4-month fetuses to 8 years of age and included females, males, and steers. There were 20 Holstein-Friesians, 23 Herefords, 9 of which were dwarfs, 7 Aberdeen-Angus, 1 Jersey, and 1 Guernsey. Of the 52 animals, 25 had intercurrent disease and 27 were normal. In addition, histological material from 11 cases of experimental hyperkeratosis in Holstein bull calves was studied.

The colons were opened immediately after collection, and the mucosa examined for gross evidence of thickening or other unusual appearance. Any such areas, with adjacent normal tissue, were removed and fixed in 10 per cent formol-saline or Bouin's fixative for histological preparation. Sections prepared from paraffin-imbedded material were stained with hematoxylin-eosin, McManus-Mowry basement membrane stain,[18] Gömöri's trichrome for connective tissues,[7] Wolbach's modification of the Giemsa stain,[9] and a hematoxylin-acid fuchsin stain. Serial sections were prepared from several blocks.

RESULTS

Normal Findings.—Two areas of mucosal thickening were demonstrated in all colons examined, with the exception of the 4-month-old fetuses. These areas apparently correspond to the Peyer's patches mentioned by Sisson.[22] One area was always related to the ileocecal valve and never involved the entire circumference of the colon (fig. 1). The second area, of variable size, was constantly found at the junction of the proximal loop and the coiled loop of the ascending colon,[10] and always involved the

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entire circumference of the colon (fig. 2, 3).

There was a circumscribed elevation of the mucosa in the ileocecal area with numerous wide gland openings (fig. 1). The colon junction area was less uniform in appearance. The mucosa of the cecum and proximal loop of the ascending colon was thin and velvety with low, circular folds, while that of the coiled colon appeared heavier and more rigid and lay in longitudinal folds. At the junction the folds were irregularly circular, thickened, broadened, and somewhat elevated (fig. 3). Multiple dilated gland openings were apparent, frequently filled with soft mucous plugs. Gross sectioning of the two areas mentioned revealed fleshy white submucosal masses producing the broadening and elevation of the mucosal folds. These masses were seen under the dissecting microscope to be mucosal gland prolongations into submucosal nodules of lymphoid tissue. By careful stripping of the mucosa from a formalin-fixed, washed colon, the "holes" occupied by these mucosal prolongations were clearly seen (fig. 4). A few scattered holes were also seen in the first part of the coiled colon.

The histological picture in the two areas was identical and similar to that described for the colon of other species[5, 24] and for the ileum of the bovine species.[6] Glandular masses were found in the submucosa of both areas. Their occurrence coincided with the presence of lymphoid tissue and was rarely found unrelated to lymphoid tissue. Numerous sections of other portions of the colon rarely contained lymphoid nodules and never showed glandular prolongations into the submucosa.

The submucosal glands presented variable types of structure but, basically, appeared to be prolongations of mucosal glands into the submucosa. In single sections, the submucosal glands frequently showed no connection with the mucosa (fig. 5), but continuity could always be demonstrated by serial sections. In some cases, the prolongation of the glands was a simple invagination with retention of the muscularis mucosae continuity but more commonly, the muscularis mucosae was stretched and thinned by the advancing prolongation and finally ruptured deep in the submucosa (fig. 6). Some areas appeared to be a primary rupture of the muscularis mucosae with extension of one or a few glands through the break (fig. 12). Frequently, all three of these processes appeared to be operating. A simple invagination of the mucosa developed secondary muscularis mucosae rupture at the farthest point of penetration and other ruptures at intermediate points. Secondary branchings of the prolonged glands were usually apparent, ramifying in all directions in the submucosal lymphoid tissue.

The epithelium of the glands in these

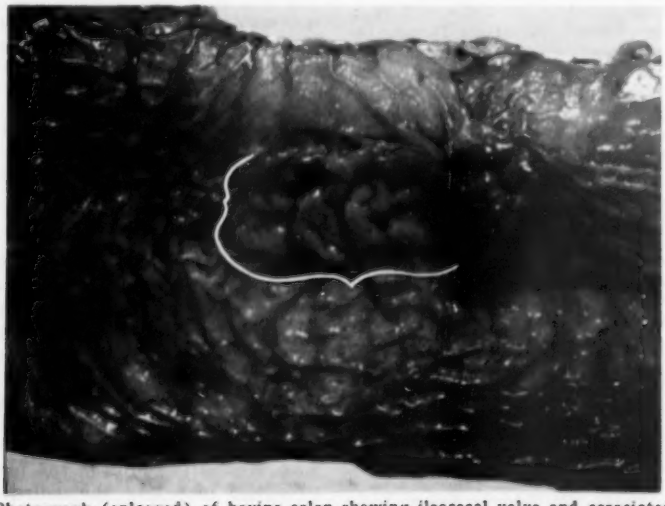


Fig. 1—Photograph (enlarged) of bovine colon showing ileocecal valve and associated Peyer's patch.

prolongations was indistinguishable from that in the uninvolved mucosa. Both columnar and mucin-producing goblet cells were present with an apparent preponderance of goblet cells in the prolonged portions. The epithelium of the deepest part of the prolonged glands, separated only by a thickened, irregular basement membrane from the lymphoid tissue (fig. 7), frequently showed atrophic changes. The goblet cells changed to columnar and then cuboidal form and, finally, assumed an endothelial or stratified mucous epithelium character (fig. 8). Pyknotic cells were often seen. The epithelial basement membranes of other portions of the gland prolongations appeared normal (fig. 9).

No significant changes were observed in the lamina propria, submucosa, muscularis, or serosa. Eosinophils were prominent in the lamina propria of most colons, and perivascular accumulations of eosinophils were occasionally observed in the submucosa.

Lymphoid tissue was variable in nature, amount, and distribution. Solitary nodules of medium and small lymphocytes were observed in the lamina propria particularly in the fetal colons. Larger nodules, with and without germinal centers, were present in the submucosa. In adult colons, and less commonly in fetal colons, the most frequent type of lymphoid nodule involved both the lamina propria and the submucosa with disruption of the continuity of the muscularis mucosae[25] (fig. 10). In the adult, this type of nodule almost invariably contained a germinal center.

The gland prolongations were intimately related to the lymphoid tissue, usually lying in the middle of submucosal nodules. Only rarely in this series was gland penetration into the submucosa observed without intimately related masses of lymphoid tissue (fig. 12). The more extensive, ramifying prolongations compressed the nodules, forming crescents of atrophic lymphoid tissue[19, 21] (fig. 11). The normally inconspicuous McManus-Mowry positive material in the lymph nodules became thickened and collapsed in these atrophic nodules (fig. 7).

Pathological Findings.—In normal animals, and particularly in those with intercurrent disease, gross and microscopic lesions were frequently found in the ileocecal and proximal loop-coiled colon junction areas. Grossly, hyperemia was common both in normal and diseased animals, standing out distinctly from the surrounding, nonthickened mucosa. Abscess-like nodules varying from 2 to 4 cm. in diameter were common in the colon junc-

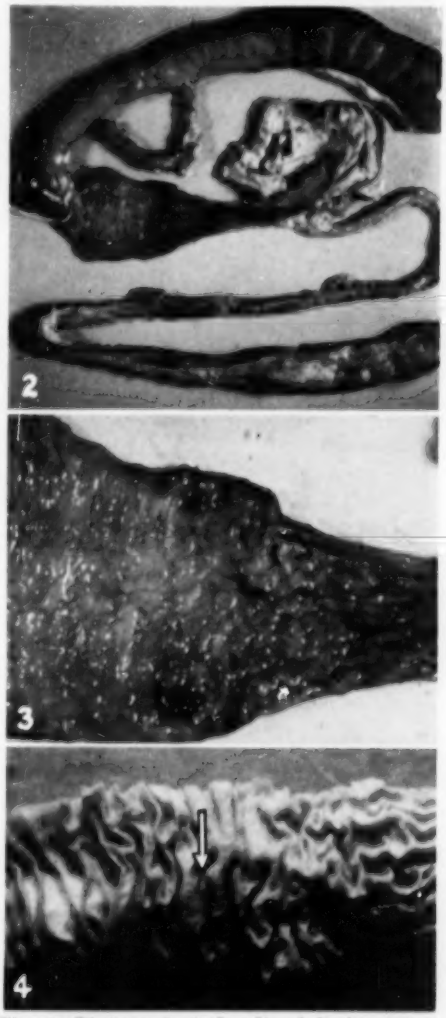


Fig. 2—Photograph (reduced) of bovine cecum, colon, and rectum. The coiled colon has been only partially straightened, and the junction of the proximal loop of the ascending colon and the coiled colon has been opened.

Fig. 3—Photograph of junction area of figure 2. The proximal loop of the ascending colon is to the left of the photograph and the coiled colon to the right.

Fig. 4—The same junction area with mucosa stripped off. The proximal loop is to the left and the coiled colon to the right. Holes (arrow) occupied by mucosal prolongations are apparent.

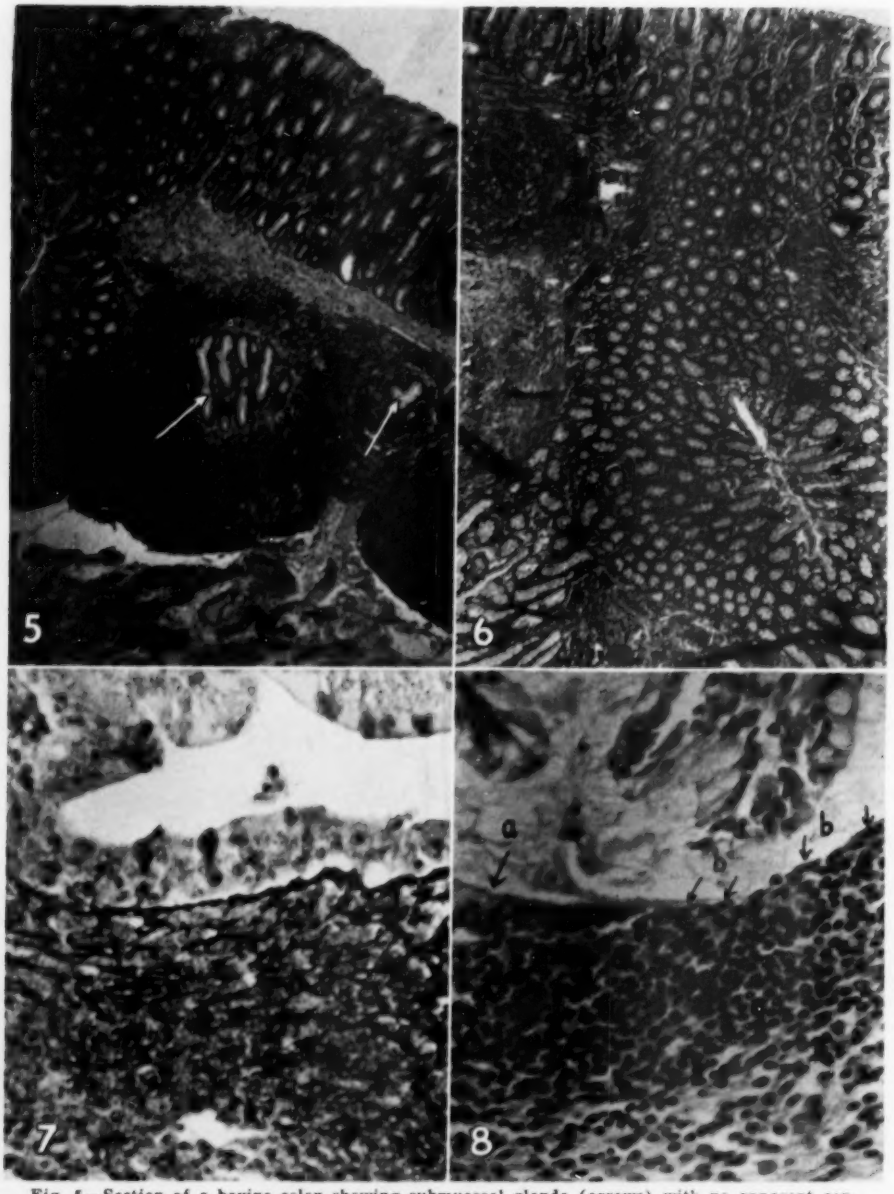


Fig. 5—Section of a bovine colon showing submucosal glands (arrows) with no apparent connection to mucosa. Hematoxylin and eosin. x 40.

Fig. 6—Section of a bovine colon showing extensive submucosal gland prolongation with loss of muscularis mucosae continuity deep in submucosa. Gömöri stain. x 40.

Fig. 7—Section of a bovine colon showing thickened, irregular basement membrane separating epithelium of prolonged glands from compressed lymphoid tissue. McManus-Mowry stain. x 240.

Fig. 8—Section of a bovine colon showing transition of columnar cells (a) to flattened, endothelial-like cells (b) in the deepest part of a prolonged gland. Hematoxylin and eosin stain. x 300.

tion area in animals with hyperkeratosis and in those with Virginia mucosal disease.[11] These nodules could be readily teased out of the mucosa and appeared to have developed within the lumens of dilated glands. *Trichuris ovis* were often found attached in the cecum and proximal loop of the ascending colon to and including the described junction area. Attached worms were never observed beyond this point in the colon.

Microscopically, the common finding in animals with hyperkeratosis or mucosal disease was cystic dilatation of the mucosal glands and, particularly, the submucosal glands with flattening of the epithelium and accumulation of mucinous, cellular debris (fig. 13). These cystic glands varied from microscopic size to grossly visible cysts which could be teased from the mucosa. Neutrophil invasion frequently converted these cysts into micro- or macroabscesses which sometimes ruptured into the lumen of the colon. These changes could not be correlated with any specific disease entity since they were found, though to a lesser degree, in the colons of a number of clinically normal animals.

Discussion

The occurrence of submucosal glands in the colons of various mammalian species has been studied by numerous workers. This study on the bovine colon, in large part, confirms the findings of these investigators. Most workers have concluded that these submucosal glands are normal structures which may undergo pathological changes. The present study demonstrated that, at least in cattle, mucosal gland prolongations into the submucosa are normal findings. Their occurrence in the colons of 7- to 9-month-old fetuses provides strong support for such a view.

While their mode of development could not be definitely ascertained, the constant relationship of the gland prolongations and lymphoid tissue indicated a possible cause–effect relationship. In some cases, the gland penetration appeared to be stimulated by the presence of lymphocytes in the submucosa without rupture of the muscularis mucosae. More commonly, damage to the muscularis by growing or migrating lymphoid tissue appeared to accompany or precede the gland penetration. The muscularis may have been damaged by lymphoid nodules penetrating from the submucosa into the mucosa or from the mucosa into the submucosa.

Maximow and Bloom[17] stated that the presence of lymphoid follicles in the sub-

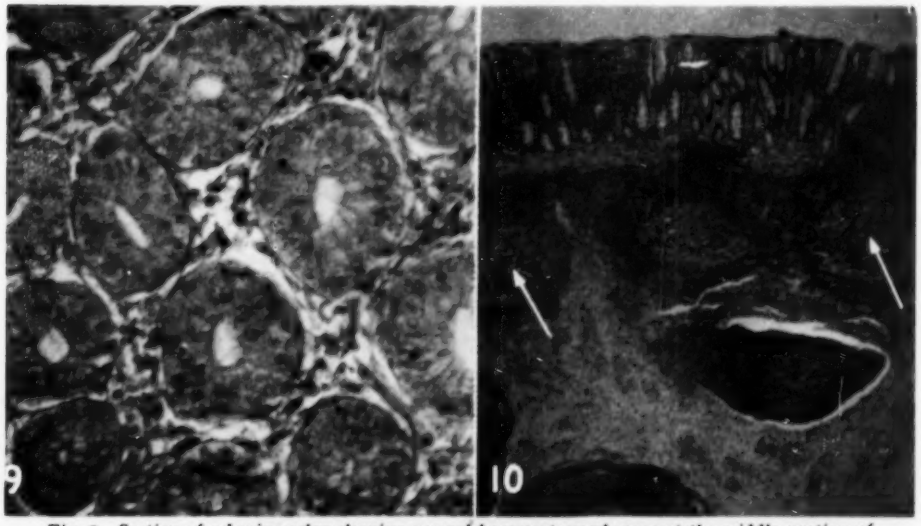
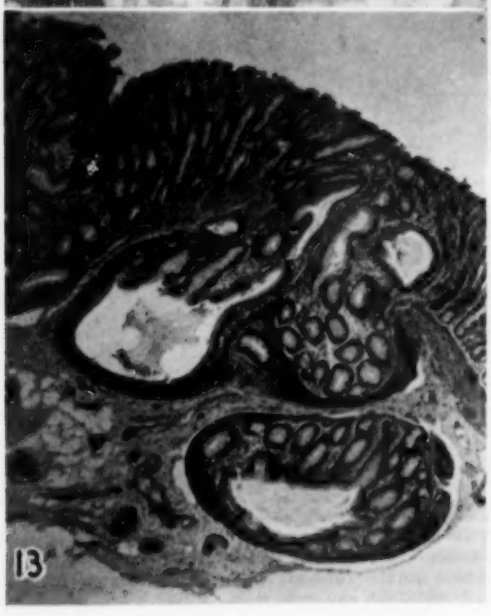


Fig. 9—Section of a bovine colon showing normal basement membranes at the middle portion of prolonged glands. McManus-Mowry stain. x 400.

Fig. 10—Section of a bovine colon with lymphoid nodules (arrows) with germinal centers involving submucosa and mucosa. Hematoxylin and eosin stain. x 32.

mucosa of the human small intestine results from expansion of follicles primarily situated in the lamina propria. Trautman and Fiebiger,[25] similarly, stated that solitary lymphoid nodules are located at the beginning of their development entirely in the lamina propria. Bargen[2] believed that lymphoid follicles arise as perivascular infiltrations, their position relative to the muscularis mucosae being determined by available space and tissue tension. Limited observations of fetal colons in this study indicated that lymphoid masses appear first in the lamina propria and extend secondarily through the muscularis mucosae into the submucosa.

Figures 14 and 15, sections of the colon of a 7-month bovine fetus, illustrate one pos-



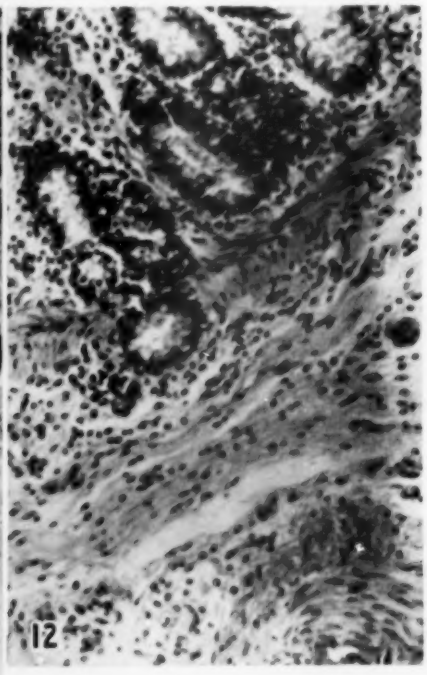


Fig. 11—Section of a bovine colon with submucosal gland prolongations producing lymphoid crescents. Hematoxylin and eosin stain. x 40.

Fig. 12—Section of a bovine colon with a solitary gland penetration of two-layered muscularis mucosa with only a few associated lymphocytes. Hematoxylin and eosin stain. x 240.

Fig. 13—Photomicrograph of dilated, cystic submucosal glands in a bovine colon from an animal with hyperkeratosis. Hematoxylin and eosin stain. x 32.

sible means of penetration of glands into the submucosa. The lymphoid tissue by either a mechanical or lytic affect weakened the muscularis mucosae, allowing the overlying glands to extend into the submucosa, pushing the thinned, attenuated muscularis mucosae before them. Eventually, the muscularis lost its continuity and only the basement membrane separated the penetrating glands from the lymphoid tissue. It would appear, then, that the essential cause for the presence of glands in the submucosa of the colon was an attenuation or destruction of the muscularis mucosae.

Most authors have considered pathological changes in the submucosal glands as secondary phenomena. This study supports such a view and, further, indicates that the cystic, abscessing changes observed are specific only for these particular structures and not for any disease entity. Both Schultze[21] and Orth[19] described cystic dilatations of the submucosal glands in cases of dysentery. Brites[4] suggested that abscesses within the wall of the human colon might be associated with submucosal gland prolongations. Follis[8] discussed the development of cystic, abscessing changes in the

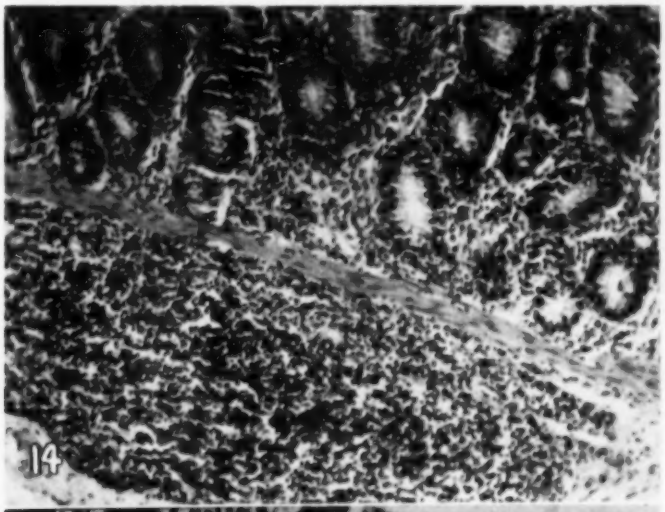


Fig. 14—Section from a 7-month bovine fetal colon with intact muscularis mucosae. Hematoxylin and eosin stain. x 240.

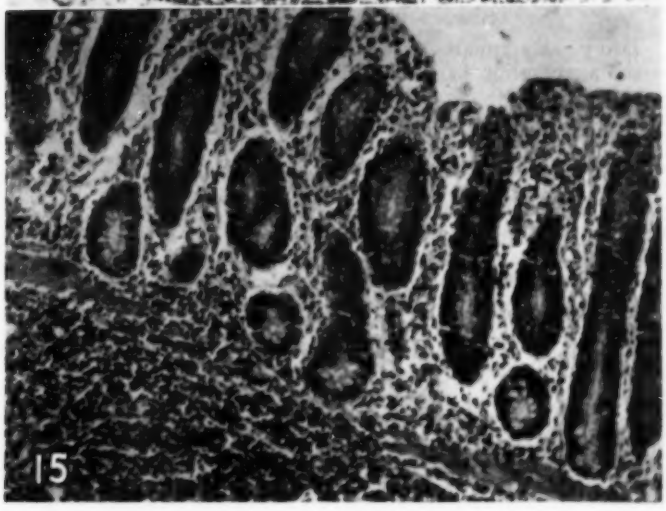


Fig. 15—Section from a 7-month bovine fetal colon with early attenuation of muscularis mucosae. Hematoxylin and eosin stain. x 240.

colon mucosal glands of human beings with pellagra and the similarity of these lesions to those produced in the mucosal and submucosal glands of pantothenic acid-deficient swine. Dunne *et al.*[5] described caseous, cystic nodules in the submucosa of pigs recovered from an experimental niacin deficiency. The chronic, cystic colitis described by Ramsey[20] in a bovine mucosal disease appeared to be localized in the proximal loop–coiled colon junction area.

Schultze[21] and Bargen[1,2] suggested that the deep-lying glands may be associated with early neoplastic changes in the human colon. The constant occurrence of such glands in the bovine colon and the rarity of carcinoma of the colon in this species[7] would indicate that further evaluation of this problem may be necessary. However, Bargen observed other changes in his series of human colons that were not found in the bovine colons.

The particular localization of lymphoid tissue in the ileocecal and proximal loop–coiled colon junction areas may be related to the relative stasis of ingesta which probably occurs at these points.[25] The lumen of the proximal loop of the ascending colon diminishes markedly at its junction with the coiled colon and a delay of ingesta passage would be expected. These lymphoid areas, therefore, may be tonsillar in nature and function. The microscopic picture of glandular crypts surrounded by lymphoid tissue is similar, though not identical, to that of the palatine tonsil.

SUMMARY

Two grossly apparent areas of lymphoid tissue aggregation have been found in the bovine colon. Microscopically, the Lieberkühn's glands in these areas showed a tendency to invade the submucosa, presenting an appearance identical to that described in man and in other animals and often referred to as the "Schultze picture." Nonspecific pathological changes have been observed in these submucosal glands in clinically normal animals and in animals with hyperkeratosis and with a type of mucosal disease.

Addendum.—Since this paper was prepared, an excellent gross description of these two lymphoid areas in the colon has been found in Ellenberger and Baum: Handbuch der Vergleichenden Anatomie der Haustiere, Springer-Verlag, Berlin, (1943): 405, 408, 448.

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Some Observations on the Bovine Corpus Luteum and Endometrium During Early Stages of Pregnancy

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IN SPITE of an increase in interest in recent years in the changes of the bovine uterus and related reproductive tissues during pregnancy, many problems remain unsolved. These include the origin of the cells lining the uterine surface and crypts (and hence the nature of the placental barrier) and the mechanism of formation of the maternal septums of the cotyledon. In the work reported here, we have attempted to obtain information regarding some aspects of these problems and have outlined some comparisons between the corpus luteum and endometrium of early pregnancy and of the estrous cycle. Such information may be useful in studies of early embryonic mortality in the bovine species.

MATERIALS AND METHODS

This study is based on observations made in 14 cows between 30 to 37 days and 1 at 66 days of pregnancy. Data on the reproductive history of the 15 cows and on the embryos recovered are given (table 1). Although some of these animals had a history of previous breeding difficulties, the current pregnancies may be considered normal since all of the cows had normal embryos at the time of slaughter. The normality of the embryos was determined by appearance and size and by the fact that the heart was beating rhythmically when the embryo was exposed in the excised uterus.

Immediately after slaughter and bleeding, the reproductive tract of each cow was removed, the uterine horn containing the embryo was opened, the embryo was inspected, and the crown-rump length was measured. Sections from the ovary containing the corpus luteum, from both the right and left uterine horns near their tips and from one or more sections of the uterine body near the bifurcation, were fixed in each of cold acetone, Carnoy's and Bouin's fluid. Routine histological processing and the histochemical determination of alkaline glycerophosphatase, glycogen, and substances positive for periodic acid-Schiff (PAS) were the same as given previously.[1] Between three to eight microscopic slides were examined in each block of tissue. An average of 45 slides were available from each cow.

OBSERVATIONS

Corpus Luteum.—The microscopic structure of the corpus luteum between 30 to 66 days of pregnancy was similar to that of the cyclic corpus luteum at diestrus. An increase in lutein cell size with pregnancy, as reported,[2,3] was not observed. In the corpus luteum of the estrous cycle, alkaline phosphatase was present at diestrus in large amounts in the endothelium

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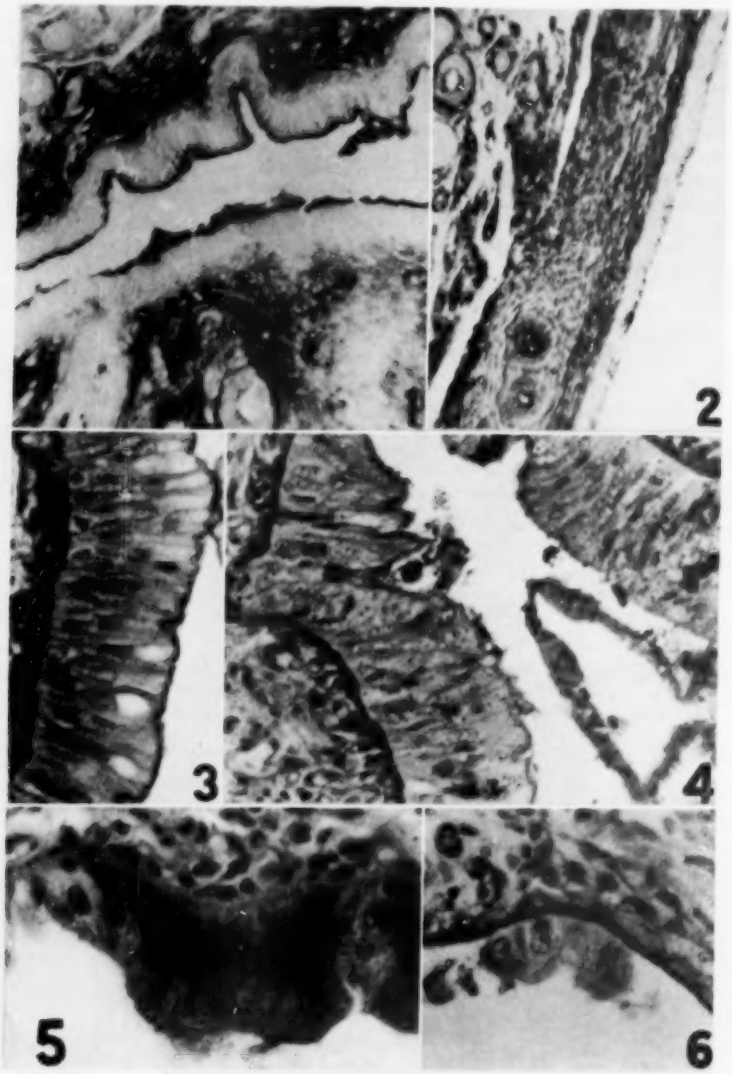
TABLE 1—Reproductive History of 15 Cows and Heifers Killed Between 30 to 66 Days of Pregnancy and the Size of Their Embryos

Breed	Age when killed (months)	No. of previous calvings	Respective services for each calving	No. of services after last calving or total No. of services in case of heifers	Days after breeding when killed	Crown-rump length of embryo (cm.)
Holstein-Friesian	30	1	3	1	30	1.0
Jersey	49	1	2	7	30	Not recorded
Holstein-Friesian	93	5	2,1,3,7,1	1	31	0.9
Holstein-Friesian	60	2	1,7	1	31	1.1
Holstein-Friesian	27	0	...	6	31	1.1
Jersey	71	3	3,1,2	2	32	1.2
Holstein-Friesian	26	0	...	10	32	Not recorded
Holstein-Friesian	86	4	7,2,1,1	5	33	1.5
Holstein-Friesian	31	0	...	6	33	Not recorded
Jersey	29	0	...	3	34	Not recorded
Crossbred	27	0	...	8	35	1.7
Holstein-Friesian	28	0	...	4	35	1.6
Jersey	50	2	1,2	8	36	1.6
Jersey	30	0	...	7	37	2.0
Holstein-Friesian	60	2	1,2	8	66	9.8

of capillaries and blood vessels, as well as in both the theca- and granulosa-lutein cells.[1] Between 30 to 66 days of pregnancy, however, high alkaline phosphatase was present only in the endothelium of arterioles. Moderate activity was present in the endothelium of capillaries, but the bulk of the lutein tissue showed little or no phosphatase activity. Granulosa-lutein cells occasionally showed slight activity in their cell borders. At the periphery of the corpus luteum and in the lutein tissue which was adjacent to the fibroblastic area lining the central lumen, moderate phosphatase activity was also present in theca-lutein cells.

Glycogen was absent from the corpus luteum of early pregnancy, as it is during the cycle. Periodic acid-Schiff positive spherules were seen in some granulosa-lutein cells during the estrous cycle but not during early pregnancy.



Figures 1–6

Endometrium.—Surface Epithelium.—The surface epithelium was usually intact at the tips of the uterine horn but was present irregularly in other parts of the uterus. The unaltered surface epithelium of the uterine horn was similar to the surface epithelium of the nongravid uterus, except that it was taller than at any time during the cycle. Many small round cells could be seen in this epithelium, presumably migrating through it to the lumen. The surface epithelium of the corpus uteri was similar to that of the nongravid uterus of comparable location. Alkaline phosphatase was present in the original surface epithelium (in both cotyledonary and intercotyledonary areas) only in the distal border which was thick and highly active in the horn (fig. 1) and thin and only slightly active in the body of the uterus (fig. 2). Glycogen was present in this epithelium in large amounts in the horn and in moderate amounts in the body of the uterus. Coarse glycogen granules were also seen in the uterine lumen, especially in the horn. Thus, the distribution of alkaline phosphatase and glycogen in the unaltered uterine surface epithelium of early pregnancy was similar to that seen a few days before and after estrus.[4]

Most of the uterine surface epithelium, however, showed either early or late stages of degeneration, complete absence of an epithelial surface, or a partial or extensive epithelial covering which was different from the original epithelial surface. Except for Björkman[5] and Hatch,[6] who claim that the uterine surface epithelium remains intact to a large extent during implantation, many investigators, including Assheton,[7] Hammond,[8] Andresen,[9] Melton *et al.*,[10] Weeth and Herman,[11] and Amoroso,[12] agree that extensive destruction of the original epithelium occurs during early pregnancy as a result of activity of the binucleated giant cells. There is less agreement, however, concerning the mechanism whereby the uterus acquires a new surface epithelium and whether this epithelium is of fetal or maternal origin.

Binucleated giant cells, which are derived from the fetal chorion as suggested by Assheton[7] and demonstrated by Wimsatt,[13] were present in the uterine lumen as isolated cells or in clusters of cells which include other fetal cells. Impending degeneration of the original surface epithelium was indicated by typical indentations of the epithelium, accompanied by apical vacuolization of the epithelial cells (fig. 3). In many instances, giant cells were anchored in such dents and, presumably, may initiate or accelerate degeneration of the epithelium (fig. 4). Some cells of the epithelium became swollen and appeared to cause neighboring cells to elongate and become compressed (fig. 5). Some of these elongated cells appeared to be forced like a wedge between the epithelium and the basement membrane which may have resulted in a loosening of the surface epithelium. Shedding of the sur-

Legends for illustrations on opposite page.

Fig. 1 — Intact original surface epithelium (intercotyledonary portion) from the anterior uterine horn with high phosphatase activity (black) in the thick distal border and absence of activity from the rest of the epithelial cells. Fixed in cold acetone and stained for alkaline phosphatase. x 113.

Fig. 2—Intact original surface epithelium from an intercotyledonary area of the uterus near the bifurcation with reduced phosphatase activity in the distal border as compared with that in the horn. Fixed and stained as in figure 1. x 113.

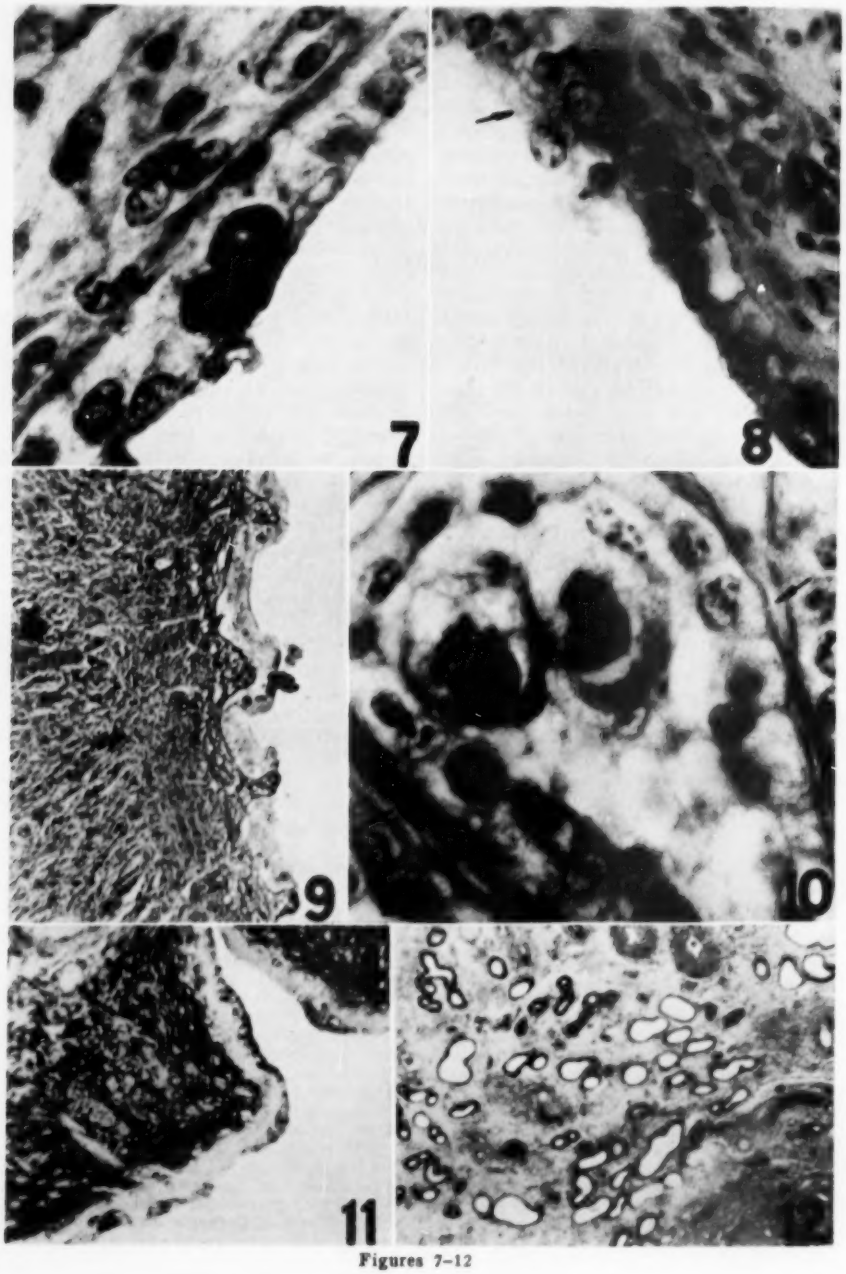
Fig. 3—Intact original surface epithelium from the anterior uterine horn with indentation and apical vacuolization of epithelial cells. Fixed in Carnoy's fluid and digested with diastase prior to staining with the periodic acid-Schiff technique. x 500.

Fig. 4—Degenerating original surface epithelium and trophoblast in the anterior uterine horn with trophoblastic elements anchored in epithelial indentation and thick basement membrance positive to periodic acid-Schiff. x 500.

Fig. 5—Degenerating original surface epithelium in the anterior uterine horn with a group of enlarged cells which cause neighboring cells to elongate and become distorted. Basement membrane does not stain with this technique. Fixed in cold acetone and stained with hematoxylin and eosin. x 500.

Fig. 6—Intercotyledonary uterine surface near the area of bifurcation with cluster of trophoblastic cells, other than binucleated giant cells, on a denuded basement membrane, which is thickened and highly positive for periodic acid-Schiff. Fixed in Carnoy's fluid and digested with diastase prior to staining with the periodic acid-Schiff technique. x 500.

face epithelium may also have been facilitated by a thickening of the basement membrane, since it could obstruct the flow of substances to and from the epithelial cells. A thick basement membrane (about $2.0 \pm 0.5\ \mu$) was always present where de-



Figures 7–12

struction and loss of the surface epithelium was evident (fig. 3, 4, and 6).*

These changes, which were initiated by the presence of fetal elements and, hence, seemed to be typically associated with the erosion of the surface epithelium during pregnancy, were different from the artifacts described for surface epithelial cells of the uterus in primates by Bartelmez [14] and in the cow by Hatch.[6]

A new surface epithelium was reestablished over denuded portions of the surface in successive stages. In intercotyledonary areas, parts of the original surface epithelium remained at this stage of pregnancy. In addition, isolated cells or, more often, clusters of cells (fig. 6) were seen on a denuded basement membrane. These clusters consisted of trophoblastic cells, some of which were binucleated giant cells. The latter were assumed to be of fetal origin. The trophoblastic origin of the remaining cells of these clusters was indicated by their close association with the giant cells and by their similarity to the cells of the fetal trophoblast. In some instances, portions of the surface were covered with a single layer of cells some of which were flattened giant cells (fig. 7). Following such epithelium along the surface, the height of the epithelium may have changed gradually to columnar or pseudostratified. Some mitotic figures were observed in cells other than giant cells. Some giant cells seemed to divide amitotically to form syncytial masses within the epithelium (fig. 8). These observations suggested that the bulk of the newly formed surface epithelium of intercotyledonary areas developed by mitosis from cells of the fetal trophoblast other than giant cells, but that in this epithelium were enclosed syncytial masses which appeared to be derived amitotically from binucleated giant cells.

The surface of the cotyledons was completely denuded of epithelium before the initial stages of crypt formation set in. But as tiny crypts were formed, groups of trophoblastic cells attached themselves to the surface and eventually gave rise to a new surface epithelium (fig. 9). Binucleated giant cells or syncytial masses derived from them have not been observed among these cells. At 66 days of pregnancy, when the cotyledons were well developed, giant cells of the fetal villi were always separated by other trophoblastic cells from maternal septums (fig. 10). Giant cells, therefore, did not appear to participate in the formation of the epithelium lining the maternal crypts.

In contrast to the original surface epithelium, the newly formed epithelium contained some alkaline phosphatase and had no distinct distal border (fig. 11). In these respects, this epithelium was similar to that of the fetal trophoblast. Some of the binucleated giant cells showed pronounced alkaline phosphatase activity. The latter contained small amounts of glycogen and,

* Thickening of basement membrane may be seen by comparing figures 3, 4, and 6 here with figures 7, 9, 10, and 11, reference 4.

Legends for illustrations on opposite page.

Fig. 7—Binucleated giant cells in the newly formed surface epithelium in an intercotyledonary area of the uterus near the bifurcation. Fixed in Carnoy's fluid and digested with diastase prior to staining with the periodic acid-Schiff technique. x 1,045.

Fig. 8—Syncytial mass (arrow) within the newly formed surface epithelium from an area similar to that of figure 7. Fixed in cold acetone and stained with hematoxylin and eosin. x 500.

Fig. 9—Initial stages of crypt formation showing cotyledonary area of uterus near the bifurcation with trophoblastic cells lining the newly formed septums of the cotyledon and the two distinct layers of the upper endometrial stroma. Fixed in Carnoy's fluid and digested with diastase prior to staining with the periodic acid-Schiff technique. x 113.

Fig. 10—Placentome at 66 days of pregnancy near the area of bifurcation with binucleated giant cells of the fetal villus separated by other trophoblastic cells from the maternal septum (arrows). Fixed and stained as figure 7. x 1,045.

Fig. 11—Newly formed surface epithelium in an intercotyledonary area of the uterus near the bifurcation with an irregular amount of phosphatase in the epithelium, absence of distinct distal border, and absence of phosphatase from the basement membrane. Fixed in cold acetone and stained for alkaline phosphatase. x 113.

Fig. 12—Lower endometrium from an area similar to figure 11 with some basal glands with wide, empty lumens and a low epithelium. Fixed in cold acetone and stained with hematoxylin and eosin. x 41.

contrary to the findings of Wimsatt,[13] some glycogen had been observed in some giant cells. The presence of material positive for PAS in the giant cells is variable between 30 to 37 days, but at 66 days of pregnancy these cells are always distinctly PAS positive.

Glands.—In the upper endometrial stroma, glands had tall eosinophilic cells with slender basal nuclei. At the apex of the cells, cytoplasmic protrusions gave off tiny droplets of secretion. In addition, many glands contained cellular debris which was probably derived from the disintegration of whole cells. Superficial glands had moderate phosphatase activity and some glycogen. However, the brush borders, which were thick and PAS positive, showed high phosphatase activity. Secretion and cellular debris, which were usually present in the lumen of superficial glands, were always highly active for phosphatase and, occasionally, also positive for PAS.

The height and eosinophilia of gland cells became progressively reduced toward the base of the endometrium. Secretion was usually absent from gland lumens. The lumen of basal glands was usually narrow, although glands which have wide, empty lumens and a low epithelium were also seen (fig. 12). As distinct from the cyclic uterus, some alkaline phosphatase was always present in the deeper lying glands and the connective tissue coat around the glands was reduced in thickness and in amount of phosphatase activity. Except for 1 animal, where glycogen was present in many basal glands, glycogen was not seen in deeper-lying glands.

Stroma.—The basement membrane of the surface epithelium was distinct and PAS positive. In sites where the surface epithelium had been shed, the basement membrane was thickened (fig. 6). Alkaline phosphatase was always absent from the basement membrane (fig. 11). The upper endometrial stroma was densely cellular, but the cell density was reduced in intercotyledonary areas as compared with that at diestrus. The cell density was higher, and the dense band was wider in the uterine horn than in the body of the uterus.

The structure of the upper endometrial stroma was different in cotyledonary and intercotyledonary areas. In addition to the greater cell density and greater width of the densely cellular band, the upper stroma of the cotyledonary area was divided into two layers (fig. 9). This division was not seen in intercotyledonary areas. The lower layer of the cotyledonary stroma contained large, thick-walled arteries and arterioles embedded in a matrix which was densely filled with small round cells.[8] The upper layer, in which were many dilated capillaries, appeared more eosinophilic as individual round cells of the matrix were increased in size and the nuclei were less dense. This increase in cell size resembled a decidual type of re-

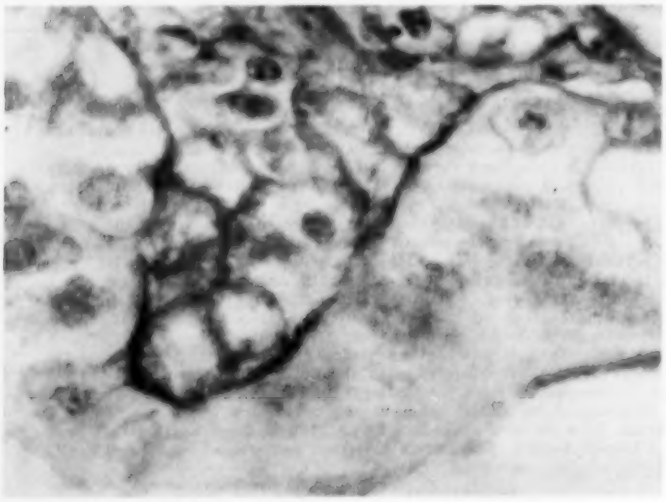


Fig. 13 — Enlarged portion of figure 9 showing periodic acid-Schiff positive material around individual cells some of which are increased in size. x 1,045.

action. The presence of small sinuses gave the tissue a spongy appearance. This layer was further characterized by the presence of PAS positive material around individual cells (fig. 13). These changes were pronounced in areas where outgrowth of connective tissue processes was taking place preliminary to formation of crypts and of maternal septums of the cotyledon (fig. 9). Apparently, it was only this upper layer which was involved in the formation of the walls of the crypts in the cotyledon, and the lower layer of connective tissue did not participate in crypt formation. In a well-developed cotyledon at 66 days of pregnancy, it was observed that the underlying dense layer of connective tissue remains intact and forms a capsule which separated the cotyledon from the loose endometrial stroma.

Just as in the nongravid uterus, alkaline phosphatase was present in the upper endometrial stroma in proportion to its cell density. In cotyledonary areas, the upper endometrial stroma lacked phosphatase except in the capillary endothelium. Glycogen was absent from the upper endometrial stroma, except that glycogen-containing cells were often present in large numbers in this layer in intercotyledonary areas. In cotyledonary areas, PAS positive patches were usually very numerous in the lower layer of the upper endometrial stroma, but intercotyledonary areas contained these patches only rarely.

DISCUSSION

It seems reasonably well established now that at least much of the original surface epithelium of the maternal cotyledons is destroyed during early pregnancy and that this destruction is caused by the action of the fetal trophoblast, especially the binucleated giant cells. However, widely differing views are held with regard to the nature and origin of the cells lining the maternal crypts which enclose the fetal villi as well as the cells lining the surface of intercotyledonary areas. A knowledge of the origin of these cells is essential to an understanding of the mechanism of implantation and of the nature of the placental barrier.

Wimsatt [13] concluded that if the surface epithelium is derived from the binucleated giant cells, it is then derived from the fetal trophoblast. He demonstrated that this is the case for the sheep but not for the cow. Amoroso,[12] however, almost simultaneously presented evidence showing that the binucleated giant cells give rise to the uterine surface epithelium in both the sheep and the cow. Hatch [6] and Drieux and Thiéry [15] consider the fetal-maternal junction epitheliochorial, hence assuming a maternal origin for these cells, but offer no proof; nor do they discuss the origin of these cells. Foley and Reece [3,4] mention only one type of cell lining the maternal crypt and, although they say that these cells are of fetal origin, they do not state from what type of fetal cell they are derived. Weeth and Herman [11] also assumed that the epithelium lining the crypts is of maternal origin but that, in the functioning placentome, the crypt lining forms part of the trophoblast and, hence, consider the placental relationship syndesmochorial. Björkman [5] stated that the uterine surface epithelium remains intact, to a large extent, during the implantation and, in addition, claims that there are two types of giant cells, trophoblastic giant cells of fetal origin and cryptal giant cells of maternal origin. Our observations suggest that, although some binucleated giant cells become incorporated into the newly formed surface epithelium in intercotyledonary areas and give rise there to syncytial masses, the bulk of the surface epithelial cells in intercotyledonary areas, as well as all of the cells lining the maternal crypts of the cotyledons, are derived from fetal trophoblast cells other than the binucleated giant cells.

With regard to the mechanism of formation of maternal septums, Jenkinson [16] states that cotyledonary crypts arise as down growth of buddings of the maternal epithelium into the subepithelial layer. Hammond [8] suggests that crypts arise by outgrowth of subepithelial connective tissue. Our observations agree with Hammond that outgrowth occurs. Furthermore, it is suggested that, preliminary to such outgrowth, a decidua-like response is normally elicited in the cells of the upper layer of the endometrial connective tissue stroma adjacent to the cotyledons. The deeper-lying region of this layer, however, does not participate in this reaction.

Alkaline phosphatase and glycogen in the uterus of early pregnancy are distributed similarly to that seen in the uterus of cows a few days before and a few days after estrus.[4] If it is assumed that the dis-

tribution of these substances in the uterus of early pregnancy is at least partly a result of progesterone secretion by the corpus luteum, the similarity of the histochemical picture in the uterus before and after estrus with that of the uterus of early pregnancy suggests that progesterone is secreted at fairly high levels by the bovine ovary both before and following estrus. This would be further evidence toward the concept that progesterone is concerned in the process of ovulation in the cow.[17]

Our observations of the corpus luteum of early pregnancy are similar to those of earlier investigators.[4] An increase in luteal cell size in pregnancy, as compared to that of diestrus,[2,3] was not seeen in our material.

The alkaline phosphatase activity of the corpus luteum of early pregnancy is in marked contrast to that of the corpus luteum during diestrus. There is little or no activity, except in arterioles and capillaries, in the corpus luteum of pregnancy, whereas all elements of luteal tissue show marked phosphatase activity during diestrus.[1]

SUMMARY

The only significant difference between the corpus luteum of early pregnancy and that at diestrus was in the amount of alkaline phosphatase activity. In contrast to the cyclic corpus luteum, the corpus luteum during early pregnancy lacks phosphatase in the lutein tissue.

Destruction of the original surface epithelium of the endometrium is preceded by indentations and apical vacuolization of epithelial cells and thickening of the basement membrane. The newly formed surface epithelium covering the maternal septums of cotyledons seems to be derived entirely from trophoblastic cells other than binucleated giant cells. In intercotyledonary areas, however, where the original surface epithelium was eroded, some binucleated giant cells may participate, in addition to other trophoblastic cells, in the formation of the new surface epithelium. In contrast to the original surface epithelium, the newly formed surface epithelium (in both cotyledonary and intercotyledonary areas) contains some phosphatase but has no distinct distal border. Some binucleated giant cells contain high phosphatase activity and variable amounts of periodic acid-Schiff positive material.

Aside from active secretion, some disintegration of whole cells in the superficial glands also occurs during early pregnancy. Moderate phosphatase activity is present in all glands, even in those basally located. Glycogen is present in superficial glands only.

The basement membrane of the surface epithelium lacks phosphatase and is highly PAS positive. The upper stroma of cotyledonary areas is divided into two layers. The upper layer has a spongy appearance, is more eosinophilic and contains PAS positive material around individual cells, some of which became enlarged. Only the upper layer seems to be involved in the formation of the walls of the cotyledonary crypts.

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The Nuclear Shift Index and Other Hematological Indexes in Surra

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SURRA is a disease caused by the hemoflagellate, *Trypanosoma evansi* (Steel, 1885). It affects chiefly horses, asses, camels, elephants, and dogs. Carabaos, pigs, cattle, and other animals which are less susceptible may serve as reservoir hosts. Monkeys, rabbits, guinea pigs, white mice, and rats have been experimentally infected with the trypanosome.

An Italian report (1946–1947) records an alleged surra infection in man in 1912. Professor Lanfranchi, while pipetting trypanosome-laden blood, accidentally sucked infective material into his mouth and through contamination of his ulcerated tonsils reportedly got the infection. However, prior to World War II, Dr. E. Y. Garcia,[2] formerly of the Institute of Hygiene, University of the Philippines, failed to infect himself and a boy through the parenteral route with hundreds of the surra trypanosome.

Of the protozoan diseases affecting livestock or poultry in the Philippines, surra is one of the most fatal and refractory to treatment. In the native horse, the disease runs an acute course and almost always terminates fatally.

At the time (1942) this work was conceived, the literature recorded no serious study of the hematology of surra. This work is limited to the study of the Schilling hemogram, particularly the nuclear shift index (NSI), and the neutrophil-lymphocyte (NLI) and the monocyte-lymphocyte (MLI) indexes or ratios.

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This paper is a condensation of a thesis submitted by the junior author in partial fulfillment of the requirements for the degree of doctor of veterinary medicine and is published with the approval of the dean, College of Veterinary Medicine, University of the Philippines, Manila.

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MATERIALS AND METHODS

Five pigs and 5 dogs were used. The pigs were selected at random from a drove artificially infected with surra trypanosomes in 1941 by de Jesus, Cabrera, and Gonzales[7] in connection with studies on the reservoir hosts of surra. The dogs, which were from the city pound, were infected with trypanosomes at the start of the canine phase of the study.

The porcine study covered the period from September 1 to Nov. 30, 1942; the canine study, from August 1 to Oct. 10, 1943, and again from February 21 to March 18, 1944.

Blood was drawn from the ear veins of the pigs daily for three months; from the dogs, until death. Either Giemsa's or Wright's stain (mostly the latter) was used in staining the blood smears.

The examination for trypanosomes was made in fresh coverglass preparation. A small drop of blood was uniformly and thinly spread under a 22-mm. square coverglass and then examined microscopically. In grading the degree of parasitemia, a system of pluses ranging from 1 to 5 was adopted as follows:

1 plus—occasional trypanosomes
2 plus—an average of one to two trypanosomes per high power field;
3 plus—an average of three to four trypanosomes per high power field;
4 plus—many trypanosomes per high power field; and
5 plus—teeming with trypanosomes.

In every case, the body temperature was taken shortly before the smear was made.

The nomenclature adopted was that of Osgood and Ashworth,[5] thus, only neutrophils showing filaments connecting nuclear lobes were classified under lobocytes or segmenters. The random method

TABLE 1—Differential Leukocyte Count in the Pig and in the Dog

Leukocyte type	Pig		Dog	
	(%)	(s.d.)	(%)	(s.d.)
Metagranulocyte	0.13±0.047	0.22±0.03	0.13±0.47	0.22±0.03
Rhabdocyte	32.87±0.04	1.86±1.20	47.26±1.75	8.20±1.20
Lobocyte	5.79±0.44	2.10±0.31	7.98±0.48	2.30±0.34
Lymphocyte	57.39±0.51	2.40±0.36	40.23±1.23	5.70±0.87
Monocyte	1.71±0.14	0.07±0.10	1.30±1.10	0.51±0.08
Eosinophil	2.11±0.01	0.61±0.01	3.10±0.23	1.10±0.16
Basophil	0.0	....	0.0	....

s.d.—standard deviation.

TABLE 2—Average Differential Leukocyte Count in 5 Surra-Infected Pigs Before, During, and After the Patent Period

Pig	M-granulocyte (%)	Rhabdocyte (%)	Lobocyte (%)	Lymphocyte (%)	Monocyte (%)	Eosinophil (%)	NSI	NLI	MLI	Temp. (C.)	Blood examination
Normal	0.13	32.87	5.79	57.4	1.7	2.1	5.7	0.68	0.03	39.2	—
Subpatent period	0.25	26.1	5.3	60.16	3.8	4.46	5.0	0.53	0.063	39.0	—Pre[5]
	0.33	26.56	4.78	59.35	3.78	5.18	5.6	0.53	0.064	39.1	—Pre[4]
	0.28	29.9	4.47	57.0	3.5	4.6	6.75	0.61	0.061	39.1	—Pre[3]
	0.34	29.96	4.36	57.3	3.8	5.0	6.95	0.6	0.066	39.0	—Pre[2]
	0.49	35.0	3.52	51.6	3.8	5.6	10.1	0.76	0.073	39.1	—Pre[1]
Patent period	0.9	43.98	1.65	42.0	5.4	6.3	27.4	1.11	0.128	39.4	1+
	1.37	44.94	1.1	43.6	4.9	6.3	42.2	1.1	0.112	39.6	2+
	1.11	42.98	1.53	40.9	5.6	7.8	28.9	1.11	0.137	39.9	3+
	0.65	45.6	1.15	35.8	7.5	9.3	39.7	1.3	0.195	40.4	4+
	1.7	42.3	1.0	40.7	6.3	7.0	44.0	1.9	0.155	40.8	5+
Subpatent period	0.46	41.66	2.18	45.6	4.7	5.3	19.32	0.97	0.103	39.1	—Post[1]
	0.49	42.1	2.65	46.7	3.6	4.8	17.07	0.97	0.077	39.2	—Post[2]
	0.35	33.16	3.53	51.2	3.4	4.8	9.55	0.72	0.066	39.1	—Post[3]

Pre[1], Pre[2], etc.,—days before patent period; Post[1], Post[2], etc.,—days after patent period.
NSI—nuclear shift index (Schilling); NLI—neutrophil-lymphocyte index or ratio; MLI—monocyte-lymphocyte index or ratio; M-granulocyte—metagranulocyte or metamyelocyte; (—)—negative for trypanosomes.

of counting recommended by Klotz[8] was used, and no less than 300 cells were examined in each smear.

The NSI is obtained by dividing the sum of the immature neutrophils with the lobocytes, the latter being taken as 1, thus an NSI of 5.7 (table 1) means that for every 5.7 immature neutrophils counted, there was one corresponding mature cell of the series or a proportion of 5.7:1. Either the MLI or the NLI is expressed as the ratio of the monocyte or the neutrophil to the lymphocyte, as the case may be.

For the normal standard in the pig, differential leukocyte counts were made on 10 healthy pigs ranging in age from 6 to 18 months; in the dog, from 10 healthy dogs ranging from 6 months to 4 years, aside from the 5 test dogs (table 1).

OBSERVATIONS AND RESULTS

The differential blood picture in the pig a few days before, during, and after the patent period is shown (table 2). These figures represent average values; for example, the white blood elements counted under a 1+ parasitemia were added as to their respective categories, averaged, and shown in the table as the differential blood picture under 1+; those under a 2+ parasitemia were similarly computed and shown in the table as the blood picture for 2+, et cetera.

The symbol pre[1] represents the average blood picture the first day before the patent period; pre[2], the second day before, et cetera; while the symbol post[1] represents the average blood picture the first day after the patent period; post[2], the second day after, et cetera.

TABLE 3—Average Differential Leukocyte Count in 5 Surra-Infected Dogs During the Prepatent and Patent Periods

Dog	Granulocyte (%)	Metagranulocyte (%)	Rhabdocyte (%)	Lobocyte (%)	Lymphocyte (%)	Monocyte (%)	Eosinophil (%)	NSI	NLI	MLI	Temp. (C.)	Blood examination
Normal	0.0	0.13	47.26	7.98	40.23	1.3	3.1	5.9	1.38	0.032	38.6	—
Preinoculation	0.0	0.21	48.8	11.3	35.2	1.95	2.75	4.34	1.71	0.05	37.8	—
Prepatent period	0.0	0.2	60.56	6.1	29.36	1.76	1.96	9.96	2.28	0.06	38.6	—Pre[4]
	0.0	0.06	62.3	3.24	29.1	1.58	3.72	13.07	2.25	0.05	38.6	—Pre[3]
	0.0	0.24	62.24	8.7	23.78	1.72	3.32	7.18	2.99	0.07	38.5	—Pre[2]
	0.0	0.2	67.32	4.96	23.76	1.34	2.30	13.6	3.05	0.056	38.8	—Pre[1]
Patent period	0.0	0.59	62.6	3.35	23.7	1.3	1.67	18.9	2.81	0.097	38.9	1+
	0.0	0.66	70.02	3.64	20.42	3.55	2.28	19.4	3.64	0.174	39.5	2+
	0.0	0.92	76.84	3.65	14.69	2.52	1.04	21.4	5.54	0.174	39.7	3+
	0.0	0.51	80.44	3.04	12.97	1.4	1.35	26.6	6.47	0.108	39.9	4+
	0.12	0.8	85.72	2.18	9.17	1.1	0.92	39.7	9.68	0.12	39.7	5+

Pre[1], Pre[2], etc.,—days before patent period.
NSI—nuclear shift index (Schilling); NLI—neutrophil-lymphocyte index or ratio; MLI—monocyte-lymphocyte index or ratio; (—)—negative for trypanosomes.

TABLE 4—Average Differential Leukocyte Picture in the 5 Surra-Infected Dogs Before Death

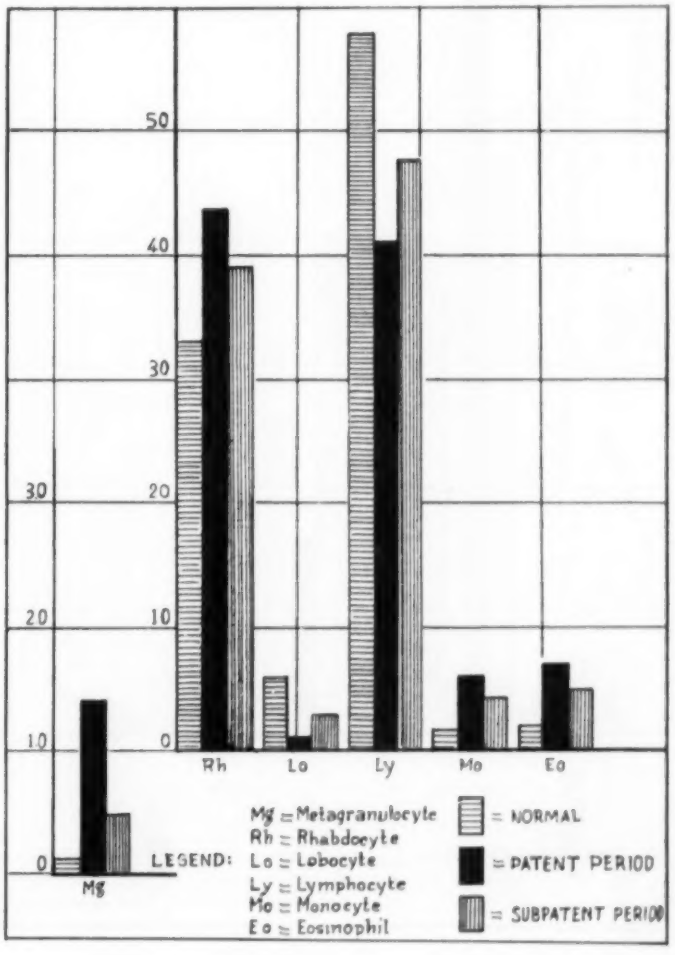
Dog	Granulocyte (%)	Metagranulocyte (%)	Rhabdocyte (%)	Lobocyte (%)	Lymphocyte (%)	Monocyte (%)	Eosinophil (%)	NSI	NLI	MLI	Temp. (C.)	Time before death
Antemortem period	0.0	0.38	76.86	2.52	15.58	2.24	2.54	30.65	5.12	0.143	39.88	3 days
	0.0	0.58	78.9	2.72	14.80	1.3	2.34	29.2	5.55	0.087	39.6	2 days
	0.06	0.84	86.52	2.92	7.84	0.96	0.68	29.9	1.15	0.122	39.36	1 day
	0.3	1.1	53.73	1.92	7.1	1.6	0.53	28.0	8.04	0.225	39.5	1-3 hours

NSI—nuclear shift index (Schilling); NLI—neutrophil-lymphocyte index or ratio; MLI—monocyte-lymphocyte index or ratio.

The differential blood picture in the dog five consecutive days before, and during, the patent period is shown (table 3). The figures in this table are computed as in table 2.

The average differential count in the dog in the three successive days preceding death and in the few remaining hours before death is shown (table 4). As in tables 2 and 3, the figures in table 4 represent average values.

A simple percentage bar chart (graph



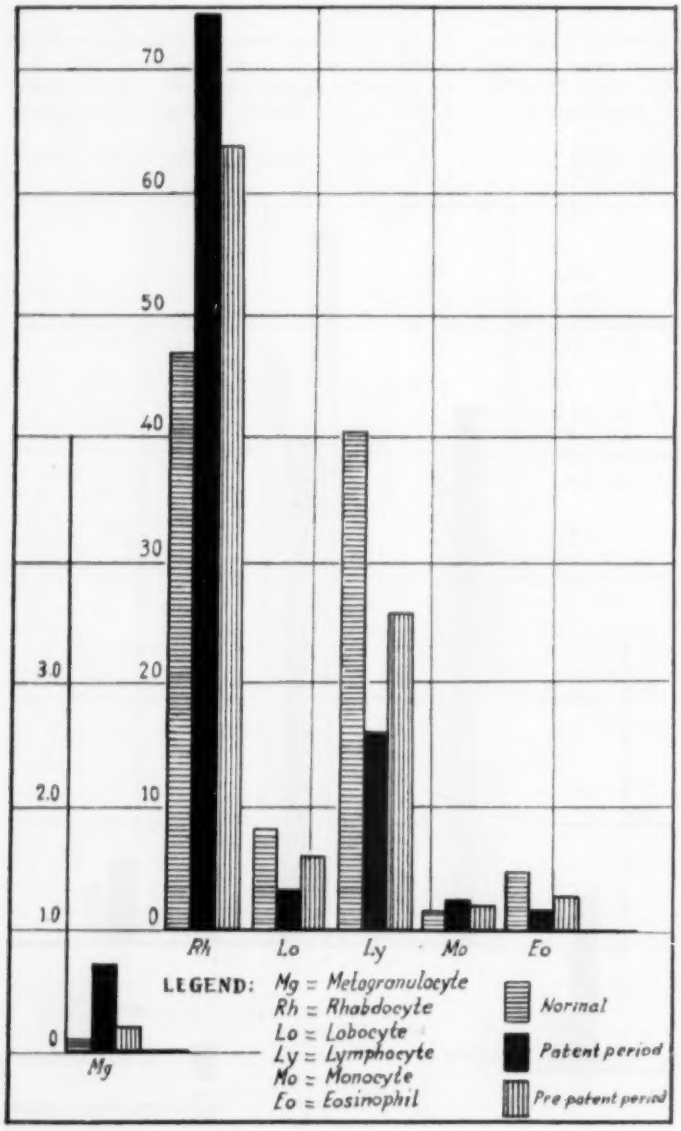
Graph 1—The differential leukocyte count in the normal and in the surra-infected pigs during the patent and the subpatent periods.

1) shows the differential leukocyte count in the normal and in the surra-infected pigs; with a corresponding one (graph 2) for the dog.

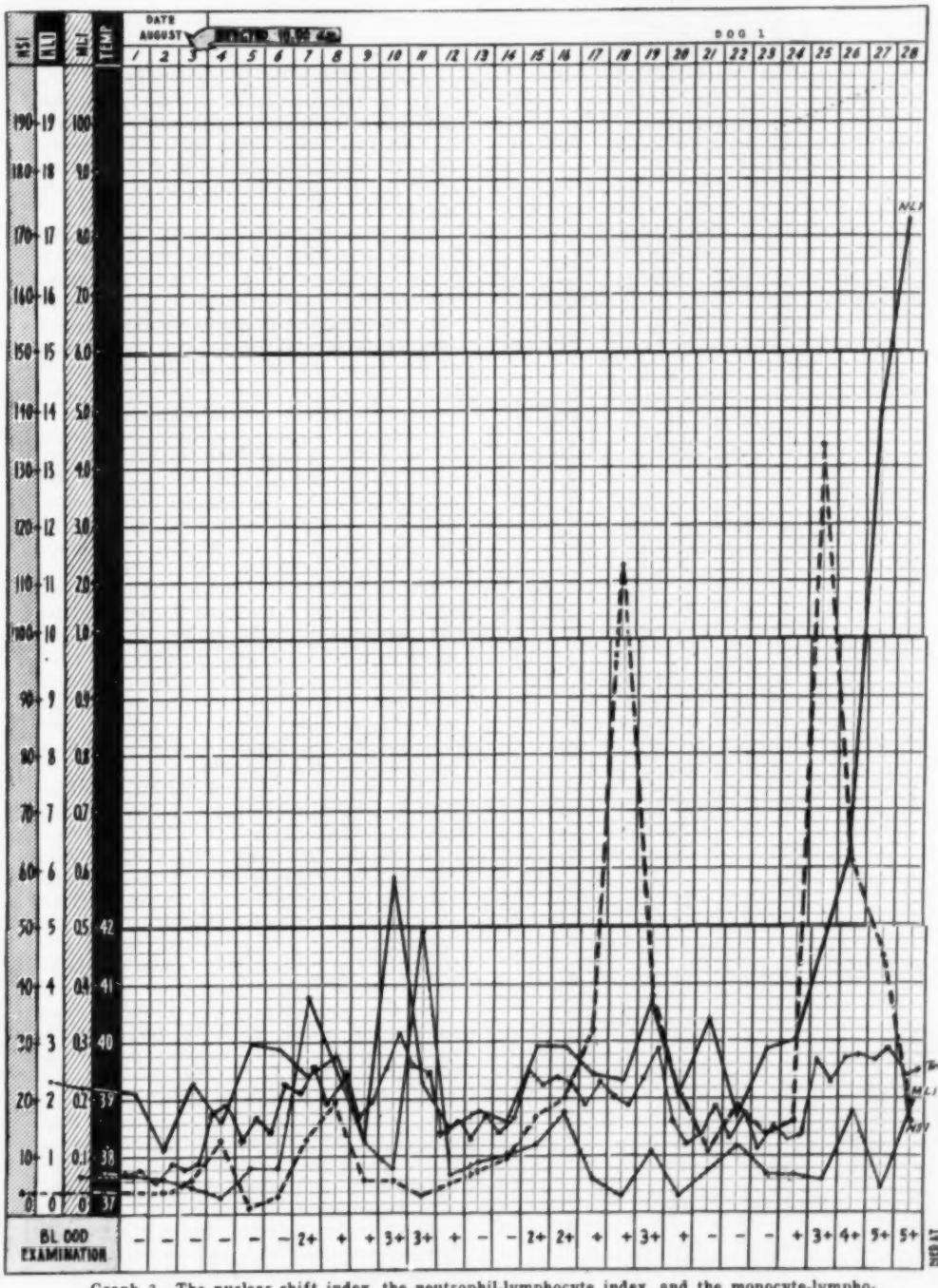
A line graph on arithmetic scalings (graph 3) shows the NSI, MLI, and NLI in surra-infected dog 1 with a corresponding one (graph 4) for pig 483.

A line graph (graph 5) shows the close relationship between the neutrophils and the lymphocytes in pig 43 and a corresponding one (graph 6) for dog 1.

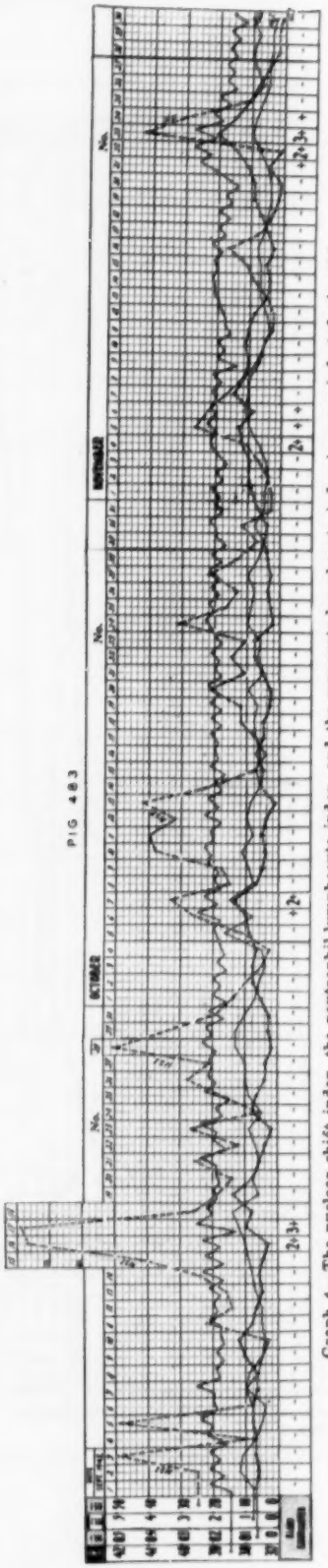
A line graph (graph 7), reconstructed from the blood findings in the 5 surra-infected dogs, illustrates in particular the NLI shortly before death.



Graph 2—The differential leukocyte count in the normal and in the surra-infected dogs during the prepatent and the patent periods.



Graph 3—The nuclear shift index, the neutrophil-lymphocyte index, and the monocyte-lymphocyte index in surra-infected dog 1.



Graph 4—The nuclear shift index, the neutrophil-lymphocyte index, and the monocyte-lymphocyte index in surra-infected pig 483.

Discussion of Results

At the termination of the study, all the pigs were living and apparently healthy. In contrast, the dogs suffered acutely from the disease and died in 4 to 32 days after the trypanosomes appeared in their blood.

The patent period in the pig has an average duration of three days with a range of from two to six days; the average subpatent period is 18.33 days.

In the dog, the prepatent period ranges from three to eight days with an average of 5.8 days; the patent period, one to 32 days with an average of 9.12 days. The average subpatent period is two days.

Using the normal Schilling hemogram for man, the normal NSI obtained in this study for either animal species may be interpreted as an exaggerated regenerative shift to the left. The figures are 5.7:1 in the pig and 5.9:1 in the dog. In man, Gradwohl [4] placed the NSI at 1:16.

Haden [5] observed that the nonfilamented cells in man do not exceed 25 per cent of the neutrophilic cells or 16 per cent of white cells of all types. In this study in the pig, the nonfilamented cells constituted 85.1 per cent of the neutrophils or 33.0 per cent of white cells of all types; in the dog, the corresponding figures were 85.6 per cent and 47.6 per cent.

The NSI, the NLI, and the MLI (tables 2 to 4) are more valuable as criteria for gauging the activity and severity of the infection than the percentage values of the different white blood elements taken individually or in combination for, even before changes in the relative proportions of the different white cell elements became apparent, these indexes were already significantly raised above the normal level.

In the pig, increase in the NSI was noticed as early as three days before the appearance of the trypanosomes in the blood; the NLI, a day before; and the MLI, even much earlier. As early as four days before the patent period in the dog, the NSI and NLI were already significantly raised above the normal, but the MLI was significantly increased only when the parasites appeared in the blood.

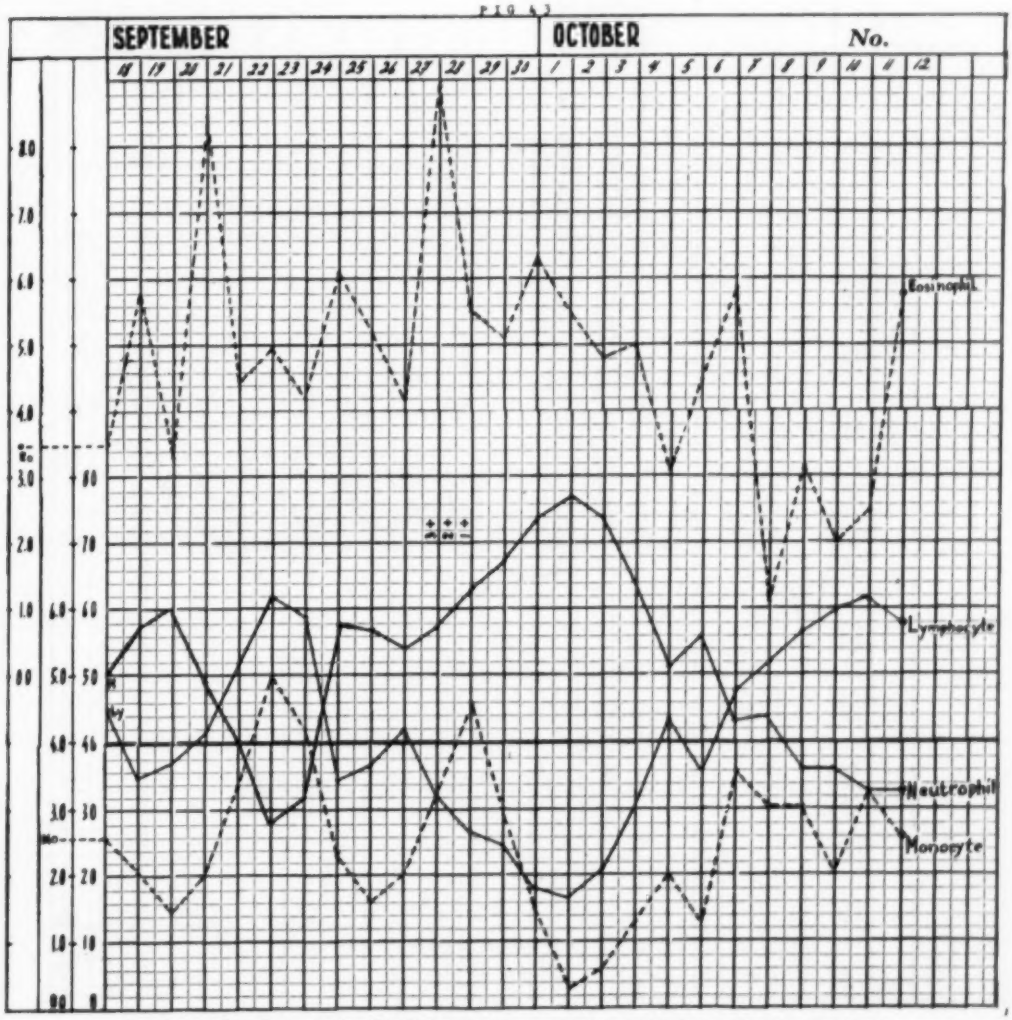
In the surra-infected dog, a neutrophil-lymphocyte ratio of 7 and above is shown to indicate a grave prognosis. Except in dog 3, in which the NLI dropped from 10.3 to 7.3, this index was found to be always

at its peak shortly before death (graphs 3 and 7).

A positive correlation between the increase in the above mentioned indexes, the degree of parasitemia, and the body temperature is apparent.

The prognostic value of the NSI in disease in human medicine is well known. Elevation of the NLI ratio, as a sign of activity of disease, has been described in tuberculosis in man. In a study on the activity of *Mycobacterium tuberculosis* infection in the guinea pig, Turley [10] observed that the NLI is highest at the onset of the disease and again at the approaching death of the animal.

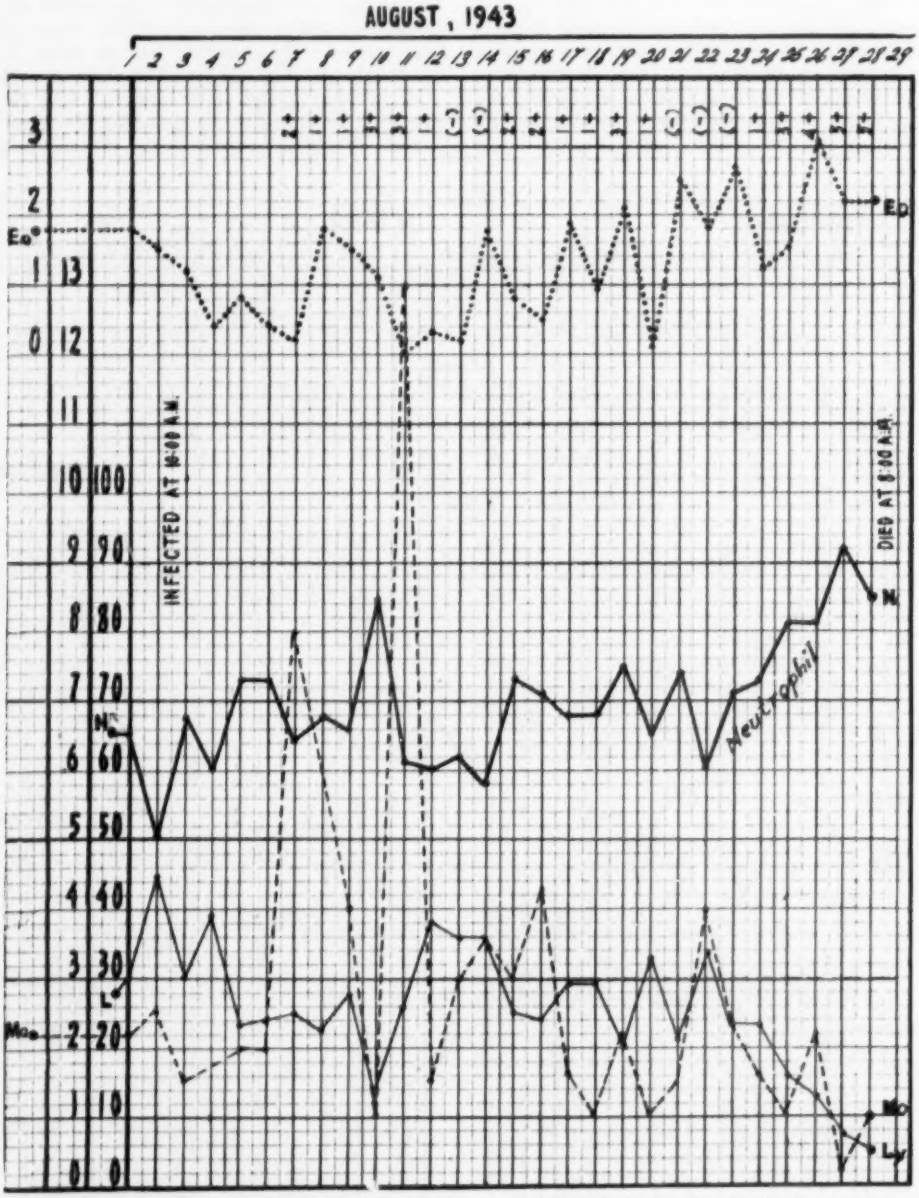
A high monocyte-lymphocyte ratio has likewise been reported in tuberculosis in man and in syphilis. Cunningham, Sabin, Sugiyama, and Kindwall [1] first called attention to the importance of this ratio in tuberculosis. Progressive lesions show a gradually increasing monocyte count and a decreasing lymphocyte count, while regressing lesions are accompanied by a reversal of these relationships. Hematoge-



Graph 5—The close relationship between the neutrophils and the lymphocytes in pig 43.

nous extension in tuberculosis, according to Osgood and Ashworth,[8] causes an increase in the monocytes while the proportion of the lymphocytes increases during healing. The same authors observed that bronchogenic extensions result in increase in the neutrophil rhabdocytes.

In syphilis, Rosahn and Pearse[9] reported that while the monocyte-lymphocyte ratio is high in untreated cases, it is



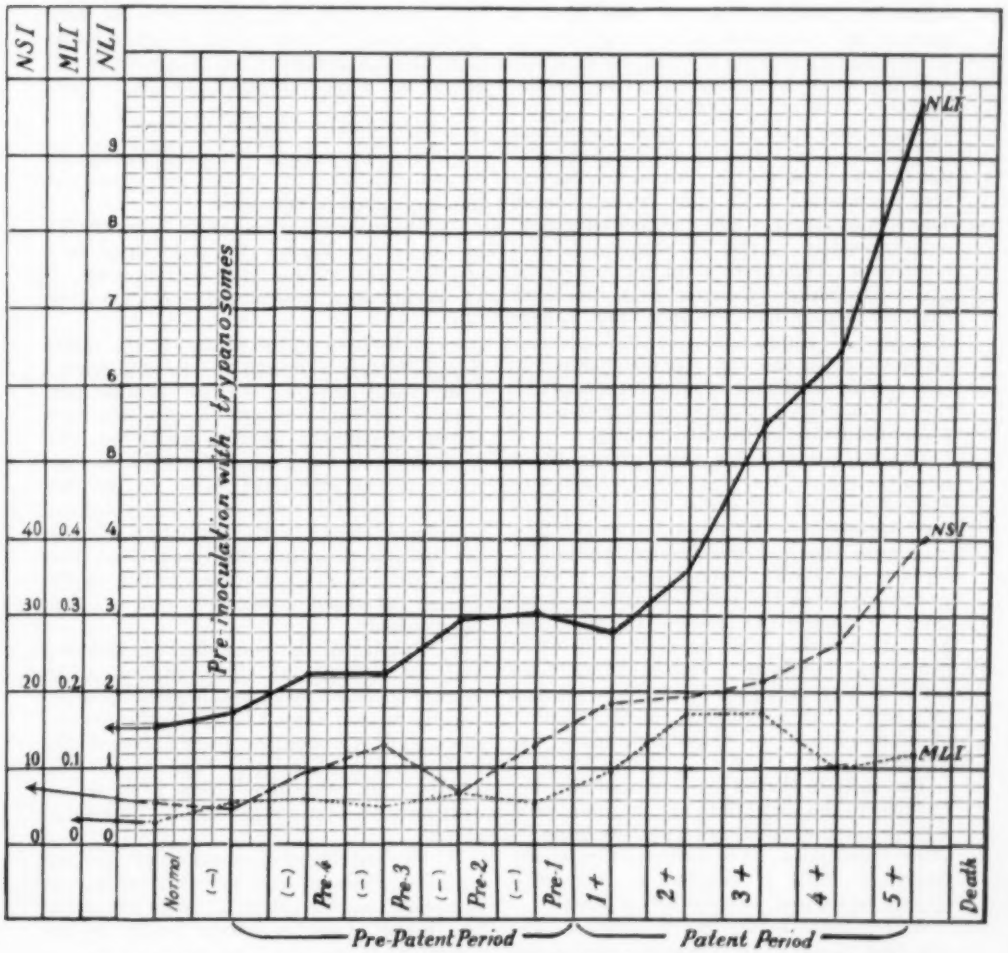
Graph 6—The close relationship between the neutrophils and the lymphocytes in dog 1.

more frequently low in persistently treated patients.

In the pig, the decrease in the lymphocytes even in the 5+ grade of parasitemia was but moderate, while the eosinophils and monocytes were increased over and above the normal and subpatent levels. This lymphocyte-eosinophil-monocyte relationship was observed in spite of neutrophilia and a continuing left shift. The increase in the eosinophilic and monocytic elements indicates that the defensive forces in the pig were strong and acting intensively. In fact, at no time during the course of the study did the pigs show any visible signs of disease.

In a prior experiment in 1941-1942, de Jesus and Cabrera [2] had found that surra in the pig runs a chronic course and may show no clinical signs other than an elevation in the body temperature at the time the organisms, which appear intermittently, are demonstrable in blood.

The reversal of the shift from left to right and the rise in lymphocytes when the parasites disappeared from the blood is a sign of recovery or of improvement. In an infection, a receding neutrophilia



Graph 7—The three average hematological indexes in the dog, particularly the neutrophil-lymphocyte index, shortly before death.

with a rising number of lymphocytes is termed, hematologically, as the "phase of cure."

Although the monocyte number was reduced when the trypanosomes disappeared, at all events this blood element remained above the normal level, showing that the pigs were resistant to the infection. Gradwohl [4] believed that a blood picture showing a persistently high shift with low counts of eosinophils and high counts of monocytes, indicated a chronic simultaneous continuance of a phase of neutrophilic struggle and of monocytic defense. The high monocyte level in the pig even in the subpatent periods, may be considered a sign of the continued presence of the infective agent, although probably in numbers too small to escape detection by the ordinary laboratory methods.

In the dog (table 4), as in all beginning infections, there was a moderate decline in the lymphocytes the first two days following inoculation with the trypanosomes. There was also a slight neutrophilia and, simultaneously, a rise in eosinophils and monocytes. This increase in the monocytic and eosinophilic elements is a defensive reaction against the pathogen. But, unlike the pig in which effective defense was maintained, in the dog (in which the disease ran an acute and rapidly fatal course), the lymphocytes receded rapidly with a corresponding rise in the neutrophils.

The parasites, being particulate, stimulated the monocytes when the trypanosomes were inoculated and again when the organisms appeared in the blood, but as the host resistance was overcome, the monocytes suffered a reduction. The eosinophils receded progressively as the infection became more severe, reaching the lowest level when the parasite number was greatest and when death was imminent. Viewed hematologically, a high neutrophilia with a marked left shift and a decrease in the number of eosinophils is of grave prognostic significance.

The shift to the left was noticed the day following the injection of the parasites. Except on the third day (pre^2 in table 3) after inoculation, this index progressively increased until it reached a peak 6.73 times the normal shortly before death.

A few hours before death (table 4), the so-called "agonal cell liberation" or "flooding" blood picture was observed, an exaggerated hyper-regenerative leukemoid shift, indicating that the bone marrow was finally overcome. This defeat of all resistance was further shown by the fact that, while the NSI remained high, the neutrophils and lymphocytes receded to low levels. While the preinoculation average neutrophil-plus-lymphocyte value was observed to be 95.6 per cent and was maintained at or about that level almost throughout the infection, this value was reduced to 64.2 per cent when death was but a few hours away.

Of all the leukocytic elements of the blood, in the resistant pig as well as in the susceptible dog, the neutrophils and the lymphocytes were observed to be very closely inter-related, either blood element presenting a graph which is a mirror image of the other (graphs 5 and 6).

SUMMARY AND CONCLUSIONS

1) A study of the Schilling hemogram in the normal and in the surra-infected pigs and dogs is discussed.

2) The normal nuclear shift index (NSI) in either the pig or the dog, using as a criterion the normal standard in man, is an exaggerated regenerative shift to the left.

3) Resistance of the species to the infection is clearly reflected in the differential white blood response. In the resistant pig, monocytosis and eosinophilia were observed during the patent periods, while the lymphocytes were but moderately reduced compared to the subpatent level. In the susceptible dog, the response to the infection manifested itself in the reduction of both the lymphocytes and the eosinophils, this reduction becoming more magnified as the disease increased in severity and as death approached.

4) Failure of the bone marrow function and the collapse of all resistance, as in imminent death, was manifested by a severe reduction (about 33%) in the neutrophils and lymphocytes percentage of the leukocytes, also, by the "agonal cell liberation" or "flooding" blood picture, an exaggerated hyper-regenerative leukemoid shift.

5) The two blood elements observed to be intimately related were the neutrophils and lymphocytes, the plotted curve of either one being almost a mirror image of the other.

6) In surra, as in tuberculosis and

syphilis, an elevated monocyte-lymphocyte index (MLI) is a sign of activity of the disease. Similarly, as in tuberculosis, a high neutrophil-lymphocyte index (NLI) indicates a grave prognosis.

7) The NSI, NLI, and MLI give clues more useful than the ordinary differential blood count from a prognostic standpoint. The increase in these indexes during the patent period compared to the prepatent and subpatent periods in either the dog or the pig is significant. These indexes may be of use as guides in ascertaining the progress and activity of the disease and in evaluating the effects of treatment.

8) In the dog, the prepatent period ranges from three to eight days with an average of 5.8 days. The disease in this animal runs an acute course and in the absence of treatment terminates fatally in from four to 32 days.

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Serological Responses of Cattle to *Vibrio Fetus* Vaccine as Measured by the Complement-Fixation Test and Tube-Agglutination Test

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As a result of the effectiveness of the preventive measures now employed against brucellosis, the occurrence of bovine and ovine vibrionic infections has become more widely recognized. The seemingly increased incidence of vibriosis has stimulated both interest and research, and the large economic losses inflicted annually by this disease emphasize the need of a rapid, reliable diagnosis, effective treatment, and prophylactic measures.

Comma-shaped organisms were first reported in aborted ovine fetuses in England by McFadyean and Stockman[1] in 1912 and were isolated from aborted bovine fetuses in the United States by Smith[2] in 1918. Smith[2] called the organism *Vibrio fetus* and little more was learned about it until the difficulties of cultivation *in vitro* were overcome during the past decade.[3] Considerable complications have been encountered by virtue of the existence of heterologous strains of the organism and the variable and transitory nature of agglutinin titers of infected animals.[1,4-9]

Intrauterine infusions of infected animals with some antibiotics have been reported, in some instances, to be efficacious in the treatment of sterility associated with vibriosis,[9-12] and two investigations on the use of *V. fetus* vaccine have been reported.[7,13] Although results of serological tests of vaccinated animals do not necessarily indicate the degree of immunity, if any, conferred by a vaccine, the responses elicited are of interest. To this end, tube-agglutination (TA) and complement-fixation (CF) tests were performed using serums obtained from 10 test heifers inoculated with *V. fetus* vaccine.

Materials and Methods

Preparation of Stock Antigen.—Vibrio fetus, strain 1236, was used in vaccine production and serological tests. It was isolated by cervical mucosal swab from a heifer in May, 1950. The organisms were maintained in a lyophilized form.[14] Seed cultures were produced by reconstitution in 10.0 ml. of Difco experimental broth 73 or 75 (DEB). After 48 hours of incubation under approximately 10.0 per cent CO_2 tension, subcultures were made by transferring 0.5 ml. of the 48-hour growth into 10.0 ml. of sterile DEB. Following a second incubation similar to the first, microscopic examinations were made and only actively motile, pure cultures were used for seeding mediums for stock antigen production.

Twenty milliliters of suitable seed culture were added to 1 liter of sterile DEB contained in a 3-liter compressed Erlenmeyer flask. Following incubation as described for seed cultures, the organisms were harvested by centrifugation and thrice washed with 0.3 percent formalized 0.85 per cent NaCl solution, pH 7.0 (formalized sa-

From the College of Veterinary Medicine, Michigan State University, East Lansing. Condensed from a thesis submitted by the senior author to the Graduate School, Michigan State University, in partial fulfillment of the requirements for the M.S. degree (1955).

Journal article 1903, Michigan Agricultural Experiment Station.

TABLE 1—Data Pertaining to 10 Heifers to Which Vibrio Fetus Vaccine Was Administered

Animal No.	Date of birth	Age at 1st inoculation (mo.)	Date Brucella M vaccine	Date *V. fetus* vaccine (amounts in ml.)		
				Oct. 8, 1954	Nov. 5, 1954	Dec. 21, 1954
1655	5/15/53	16¾	12/29/53	1	2	2
1656	5/21/53	16½	12/29/53	1	2	2
1663	6/26/53	15½	12/29/53	1	2	2
1666	7/22/53	14½	12/29/53	1	2	2
1668	7/31/53	14¼	12/30/53	1	2	2
1682	10/5/53	12	7/16/54	1	2	2
1685	10/15/53	12¼	7/16/54	1	2	2
1688	11/3/53	11	7/16/54	1	2	2
1702	1/3/54	9¼	.....	1	2	2
1707	1/24/54	8½	.....	1	2	2

line). The washed cells were suspended in a suitable volume of formalized saline to constitute the stock antigen and were stored at 4 C.

Vaccine Preparation and Administration to Test Animals.—Vaccine was prepared by adjusting the concentration of the stock antigen to approximately 50×10^8 cells per milliliter, using formalized saline as the diluent. Ten Holstein-Friesian heifers were selected for vaccination. The test animals ranged in age from approximately 8 to 17 months and had been found to be negative to the Brucella plate-agglutination test. Three subcutaneous inoculations were made in either the right or left cervical region. One milliliter of vaccine was given at the primary inoculation, and 2.0 ml. at the second and third inoculations. The second inoculation was made four weeks and the third was made ten weeks following the first (table 1).

Blood samples were collected from all animals at the time of initial inoculations and periodically thereafter for approximately nine months. Serums were harvested individually and stored in stoppered, sterile milk vials at —40 C. before and after serological testing.

Tube-Agglutination Test.—Stock antigen was diluted with formalized saline to yield 76.0 per cent light transmission through the red filter of a Cenco industrial-type photelometer. In the TA test, 2.0 ml. of adjusted antigen were placed in the first of a series of tubes, and 1.0 ml. in each succeeding tube. In the first tube, 0.08 ml. of serum was placed, giving a serum dilution of 1:25. Serial twofold dilutions were made by pipetting 1.0 ml. of serum-antigen mixture to the next tube of antigen in the series. Control tubes were included in all TA tests.

Following 48 hours of incubation at 37 C., the highest serum dilution in which there was a perceptible agglutination was recorded as the agglutinin titer (AGG titer).

Complement-Fixation Test.—The usual techniques of complement fixation (100 %) were employed. Buffered 0.85 per cent saline[10] was used as diluent. A 2.0 per cent suspension of sheep's red blood cells (2 % r.b.c.) was prepared daily

TABLE 2—Individual and Average Serum Titers, as Measured by the Tube-Agglutination and Complement-Fixation Tests, of 10 Heifers in Response to Vibrio Fetus Vaccination

Date	Test	No. of heifer										
		1655	1656	1663	1666	1668	1682	1685	1688	1702	1707	Av.
10/8/54*	AGG	0	0	0	0	200	100	0	25	0	0	32.5
	CF	0	0	0	0	0	0	0	0	0	0	0
10/15/54	AGG	400	200	400	200	800	400	800	200	400	400	420.0
	CF	10	5	5	1	10	10	10	1	20	40	11.2
10/22/54	AGG	800	800	800	800	400	800	800	200	400	400	620.0
	CF	160	40	80	160	80	160	160	80	320	640	188.0
10/29/54	AGG	400	400	400	800	400	800	400	200	400	200	440.0
	CF	320	80	160	320	160	640	320	160	320	1,280	376.0
11/5/54*	AGG	200	400	200	400	400	400	200	200	400	200	300.0
	CF	320	160	640	1,280	320	1,280	640	160	320	1,280	640.0
11/12/54	AGG	3,200	1,600	800	1,600	800	800	800	800	1,600	3,200	1,520.0
	CF	1,920	1,280	1,920	2,560	1,280	3,200	1,920	1,920	2,560	3,840	2,240.0
11/19/54	AGG	3,200	800	1,600	800	1,600	1,600	800	400	800	800	1,240.0
	CF	2,560	1,280	1,280	1,920	1,280	3,200	1,920	1,280	2,560	3,840	2,112.0
11/26/54	AGG	1,600	†	400	800	800	400	200	†	400	800	675.0
	CF	2,560	†	1,280	1,920	640	2,560	1,920	†	2,560	3,200	2,080.0
12/3/54	AGG	800	400	400	400	200	400	400	400	200	800	440.0
	CF	2,560	640	1,280	1,280	640	2,560	1,920	640	1,920	3,200	1,664.0
12/10/54	AGG	800	200	400	400	200	800	200	200	50	400	365.0
	CF	2,560	640	1,280	640	640	2,560	1,280	640	1,920	2,560	1,472.0
12/21/54*	AGG	400	400	400	400	400	800	400	400	100	400	410.0
	CF	3,200	640	640	1,280	640	1,920	1,280	640	1,920	2,560	1,472.0
12/31/54	AGG	3,200	3,200	1,600	3,200	1,600	800	800	800	3,200	6,400	2,480.0
	CF	5,120	3,840	3,200	3,840	3,840	5,760	3,200	2,560	5,120	3,840	4,012.0
1/14/55	AGG	200	800	400	1,600	1,600	400	400	800	1,600	1,600	940.0
	CF	3,200	1,920	1,920	2,560	1,280	3,840	1,280	640	3,200	1,280	2,112.0
1/28/55	AGG	200	400	400	1,600	800	200	400	1,600	1,600	800	820.0
	CF	640	160	320	640	160	1,280	160	80	1,280	320	504.0
2/10/55	AGG	100	400	400	400	200	200	200	400	400	800	350.0
	CF	160	80	80	160	80	320	40	40	160	80	120.0
2/28/55	AGG	200	200	200	200	400	100	100	200	400	400	240.0
	CF	80	20	40	80	40	80	20	40	40	20	46.0
3/11/55	AGG	100	†	200	100	200	100	100	200	200	100	144.0
	CF	40	†	10	20	10	20	5	10	20	5	15.0
3/25/55	AGG	100	†	200	200	200	100	200	200	200	200	177.0
	CF	10	†	5	1	1	5	1	1	1	5	3.0
4/22/55	AGG	100	†	200	†	200	100	100	100	100	100	125.0
	CF	5	†	1	†	‡	1	1	‡	1	5	2.0
6/24/55	AGG	25	†	†	100	100	25	100	50	100	†	71.0
	CF	+	†	†	+	+	+	+	+	+	+	+

* Dates of inoculations; † test not performed; ‡ sample anticomplementary; + complement fixed only with undiluted serum.

from whole blood stored at 4 C. in an equal volume of modified Alsever's solution.[15] Commercially prepared hemolysin* was titrated with fresh guinea pig serum. Pooled lots of complement obtained from no less than 5 guinea pigs were quick-frozen, hermetically sealed, and stored at —40 C. Stock antigen was adjusted to a density corresponding to that of McFarland nephelometer tube No. 3.

The minimal hemolytic dose (m.h.d.) of complement and hemolysin was determined employing the necessary controls of all reagents. Individual inactivated serum samples collected from test animals were tested according to the following procedure. A mixture of 1.0 ml. of saline solution, 0.1 ml. of antigen, 0.1 ml. of complement (2 m.h.d.), and 0.02 ml. of serum (diluted or undiluted) was incubated at 37 C. for 30 minutes. The incubation was repeated following the addition of 0.1 ml. of hemolysin (3 m.h.d.) and 0.5 ml. of 2 per cent r.b.c.

The highest dilution of serum with which there was complete fixation of the complement upon completion of the second incubation was recorded as the complement-fixation titer (CF titer).

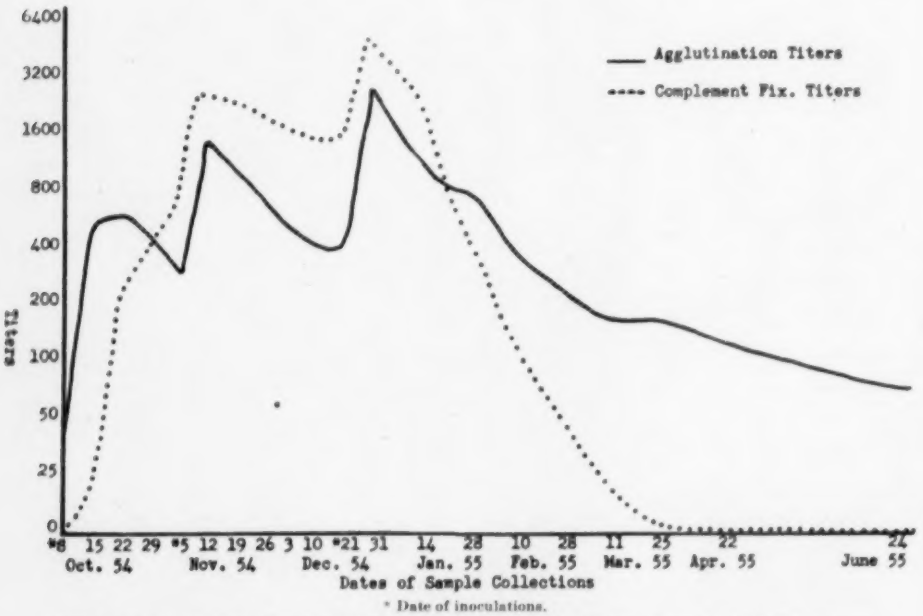
RESULTS AND DISCUSSION

All test animals responded to the administration of the vaccine by the development of agglutinins and complement-fixing antibodies as recorded (table 2). A graphic representation of the average titers is shown (graph 1).

The relation of the AGG titer to the CF titer is not known but, as observed, the AGG titer developed more rapidly than the CF titer in response to the primary injection. This might indicate that the CF test may be found to have less value than the AGG test as a diagnostic aid in the early stages of natural infections.

As shown by both tests, injections made after the primary inoculation produced a rapid and continuous increase in titer in all animals for approximately seven to ten days. With the exception of the CF titer following the original injection, this period of rising titer was immediately followed by a marked decrease; the AGG titer declined from an average of 2,480 at the last and highest peak to an average of 125 approximately three months after the final inoculation, the CF titer fell from an average of 4,012 at the highest peak to a point where complement was fixed by an

* Anti-sheep hemolysin glycerinized, rabbit, Sharpe and Dohme.



* Date of inoculations.

Graph 1—The average tube-agglutination and complement-fixation titers of 10 heifers inoculated with Vibrio fetus vaccine.

average serum dilution of 1:2. A similar transitory agglutination titer occurred subsequent to immunization with an avianized vaccine.[7]

Titers obtained from serum samples collected from individual test animals on the same date varied considerably. As an average, the AGG titers varied by four dilutions from the highest to the lowest titer of the same date, while CF titers exhibited a slightly greater range. This individual variance has been observed in natural infections and has been attributed in part to the occurrence of different antigenic strains of the causative organism within the same herd.[5] The titers obtained in this experiment emphasize the variability of responses on the part of individual animals since a homologous strain of *V. fetus* was employed.

Cross-agglutination reactions with Brucella have been observed.[16] However, the test animals were found to be negative to Brucella plate-agglutination tests prior to primary inoculations. In addition, CF tests in which *Brucella abortus* antigen was substituted for *V. fetus* antigen were made on all test animals at the time of initial inoculations and four weeks thereafter. No fixation of complement occurred, thus eliminating the possibility that anamnestic Brucella reactions were being encountered.

The significant development of agglutinins and complement-fixing antibodies by all test animals indicates the need of further investigations. The authors do not suggest that the serological reactions obtained from vaccinated animals indicate any degree of immunity because the resistance of the animals could not be tested under the experimental conditions. The significance of the serological responses elicited by the vaccine has not yet been established.

SUMMARY

Ten heifers, approximately 8 to 17 months old, were inoculated with a formalized *Vibrio fetus* vaccine (50×10^9 cells/ml.). Three subcutaneous injections in the cervical region were made, the first (1.0 ml.) was followed in four weeks by a second (2.0 ml.) and a third (2.0 ml.) was given six weeks after the second.

Blood samples were collected periodically from all animals for approximately nine months after the initial inoculation. Serum titers for *V. fetus* antibodies, determined by means of the complement-fixation and tube-agglutination tests, indicated a marked response during a ten-day period subsequent to each inoculation, with higher titers after each successive inoculation. Titers were transitory and generally declined after the tenth postinoculation day.

Any protection afforded the test animals and the significance of the serological responses, if any, have yet to be determined.

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Observations on the Morbid Anatomy and Histology of Manchester Wasting Disease of Cattle in Jamaica, and Related Conditions in Other Countries of the Americas

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MANCHESTER wasting disease is a chronic disease affecting cattle in certain areas of Jamaica, B.W.I. Clinically, the condition is characterized by a progressive wasting, stiffness of the joints, and symptoms associated with impaired circulation. A description of the circumstances under which the syndrome occurs, the clinical signs, and some of the gross pathological changes have been given by Arnold and Fincham [1] who quoted 13 references to similar, and possibly related, conditions.

A similar condition has been recorded as occurring in other parts of the tropical world and a resumé of this literature is given. The object of this paper is to describe in greater detail the histological findings, to compare these with the findings in other countries, and to attempt to interpret them in terms of a disease process.

REVIEW OF LITERATURE

The earliest full account of a similar disease was given in 1912 in Argentine by Lignieres [2] who described a condition known as "enteque" which he considered due to a chronic pasteurellosis. His description of the disease was widely quoted in textbooks for the next 20 years. The term "enteque" is still used in Argentine and Brazil to describe any chronic wasting condition. It is now subclassified as "enteque diarrhetico" (caused by parasitic infestation) and "enteque seco" (of unknown origin). Recent observations have failed to confirm earlier suggestions of an infection in either condition. Rosenbusch [3] has provided a full description of enteque seco as it is encountered in the Argentine Republic and a report on the pathology and circumstances surrounding the occurrence of the disease. The disease is of considerable local importance in parts of Buenos Aires province, and on one farm visited in April, 1955, over 200 affected animals were seen.

Enteque (seco) has been described in cattle in Brazil by Pardi and dos Santos,[4] who gave a brief description of the gross and microscopic necropsy findings. These authors stated that the disease is associated with particular parts of the Matto Grasso area of Brazil and considered that the condition was in some way connected with the soil type. Of 2,172 animals entering a particular slaughter house, 114 (5.24 %) were found to be affected to the extent that there was ossification of the lung, and 635 (29.2 %) showed gross renal changes. Other authors such as Pires,[5] Vasconcelos,[6] Mucciolo,[7] and Tibirica [8] have described similar lesions affecting the lung or the aorta.

In Hawaii, a similar condition has been described by Hendershot,[9] and sections of lung from Hawaiian animals have been examined by one of the authors. Known as "naalehu disease," it has long been recognized as being associated with grazing in particular parts of the islands.

Other similar lesions have been recorded in isolated animals in other countries including Germany, Scotland, Switzerland, and the United States, but such accounts have not associated the lesions with a particular disease process or with animals kept in any particular area or environment. Similar lesions have also been reported by various authors as having occurred in various experimental animals.

MATERIAL AND METHODS

Diseased animals were brought to the laboratory for a complete necropsy which included opening of all cavities. Material from various organs was fixed in formalin and, occasionally, in Helly's bichromate mixture. Paraffin blocks were prepared and the sections stained with haematoxylin-eosin and, when necessary, with Mallory's trichrome, Kornhauser's elastic tissue, and Gömöri's iron stains. Material from 19 animals was examined as a basis for this paper.

RESULTS

MORBID ANATOMY

The general picture of Manchester wasting disease was one of chronic emaciation (fig. 1). The carcasses were "hide-bound," the coat dull, with a rough and faded appearance, and decubitus ulcers from prolonged recumbency were frequently present.

At necropsy there was generally an ab-

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sence of body fat, making subcutaneous and intramuscular fibrous tissues unusually prominent. This was accompanied, in some cases, by a generalized edema or ascites or both. Small blood vessels encountered in the tissues were often calcified. In a few animals, small scattered areas of muscle appeared edematous and pale pink, similar in appearance to muscle of animals affected with "white muscle disease" (muscular dystrophy, avitaminosis E).

Circulatory System.—The coronary fat was often replaced by edematous fluid (serous atrophy) and there was sometimes calcification of coronary blood vessels on both sides of the heart. In some cases, the right atrium showed calcified deposits in the endocardium, more particularly around the opening of the vena cava, but although the right ventricle was frequently hypertrophic it was never found to show calcium deposits. In many instances, the left side of the heart showed extensive calcification, the ventricle being particularly affected and the process affecting the auricloventricular and semilunar valves and extending into the aorta. The apex of the ventricle was affected least of all. The aorta was calcified and, in severe, long standing cases, the deposits sometimes extended throughout the entire length. This resulted in the formation of a rigid inelastic tube with distortion, due to the irregular projection of calcified or bony plates into the lumen (fig. 2). In less advanced cases, lesions were restricted to the bulb of the aorta and to such sites as the iliac bifurcation or the origin of the renal and carotid arteries.

Calcification of large and medium-sized arteries was usually found but varied in extent and situation. Calcified plaques were sometimes present in the jugular veins, or venae cavae (fig. 3).

Respiratory System.— Excessive pleural fluid was present in some instances. On the pleura, small areas of calcific deposition were sometimes present, especially on the diaphragm. In animals which died suddenly, petechial hemorrhages were sometimes found in similar situations. The lungs were affected in varying degrees. In advanced cases, parts of the lungs, more usually the posterior borders, failed to collapse when the thorax was opened and were found to contain areas of calcification (fig. 4). Such areas, sometimes as much as two-fifths of the lung, on palpation felt like a "loofah" sponge. These calcified areas,

Fig. 1—A 4-year-old heifer affected with Manchester wasting disease. Note slightly flexed knees and straight hocks. The arched back and general emaciation are typical.

where lacunae up to 5 mm. in diameter were found, were surrounded by wide bands of emphysema, especially between lobules, and by areas of congestion. Although carnified areas resulting from past pneumonic changes were sometimes seen, active pneumonia was not commonly present. Lung worms and resulting changes were observed but were not commonly present.

Urinary Systems.—The kidneys usually showed areas of scarring extending into the medulla, resulting in a local depression of the cortical surface. In some kidneys, the medulla was poorly defined. Retention cysts containing crystals of triple phosphate and calcium carbonate were seen in some.

The bladder usually showed no abnormalities but occasionally, when associated in life with hematuria, calcified deposits surrounded by areas of recent hemorrhage were found in the mucosa.

Digestive System.—The liver was normal in size and appearance in most instances but calcified deposits were found on the wall of the abomasum and in the mesentery of a few. The mesenteric arteries were commonly calcified.

Spleen.—The spleen sometimes showed areas of calcium deposits in the capsule or trabeculae.

Skeletal System. — The joints of both fore limbs and hind limbs, especially the weight-bearing joints, showed progressive lesions terminating in destruction of the articular surfaces. These became roughened, soft, and chalky, especially around areas of ligamentous attachment. Eroded areas up to 20 mm. in diameter appeared in the articular surfaces, extending through the cartilage to the underlying softened bone (fig. 5). There was little or no inflammatory reaction or pus formation in association with these lesions, which were considered to result from occlusion of terminal arterioles. The bones were normal in appearance, texture, and hardness. Chemical analysis showed an apparent increase in the percentage of phosphate over the amounts present in normal bone.

The tendons, especially in the legs but also those elsewhere, and such elastic tissues as the diaphragm, showed discrete areas of calcification. The calcification of the ligaments of the legs appeared to cause pain and to restrict movement.

Fig. 2—Gross aspect of left side of heart and aorta of heifer showing rugosity of aorta due to calcific deposits. The semilunar valves are thickened and rigid and there are calcific deposits in the atrium and on the cordæ tendineæ and wall of the ventricle.

HISTOLOGY

Heart.—There was thickening and calcification of the endocardium and subendocardial tissues which was unrelated to coronary blood vessels and not secondary to degeneration of myocardium, such as may result from embarrassment of the coronary circulation. In some sections of the mitral valve, there was diffuse calcification. The myocardium showed areas of hyalinization and interstitial inflammatory reaction, with collection of mononuclear cells and edema in some sections. Sarcocysts were present in most sections (fig. 6).

Arteries.—Aorta.—There was a marked calcification of the intima extending into the media and in places the internal elastic membrane was disrupted. In the affected area, the pre-existing elastic fibers were fragmented or destroyed while some new fibers had been formed diffusely throughout and surrounding the area.

Medium-Sized Arteries (Carotid, Hepatic.—Again there was calcification of the intima and of the media with damage of the internal elastic membrane which was fragmented and interrupted. However, in certain areas there was also a swelling of the intima and partial occlusion of the lumen. In certain sections, the calcific deposits in the media appeared to be older than those of the intima (fig. 7).

Fig. 4—Gross aspect of part of border of lung of heifer showing calcification (see fig. 8 and 9).

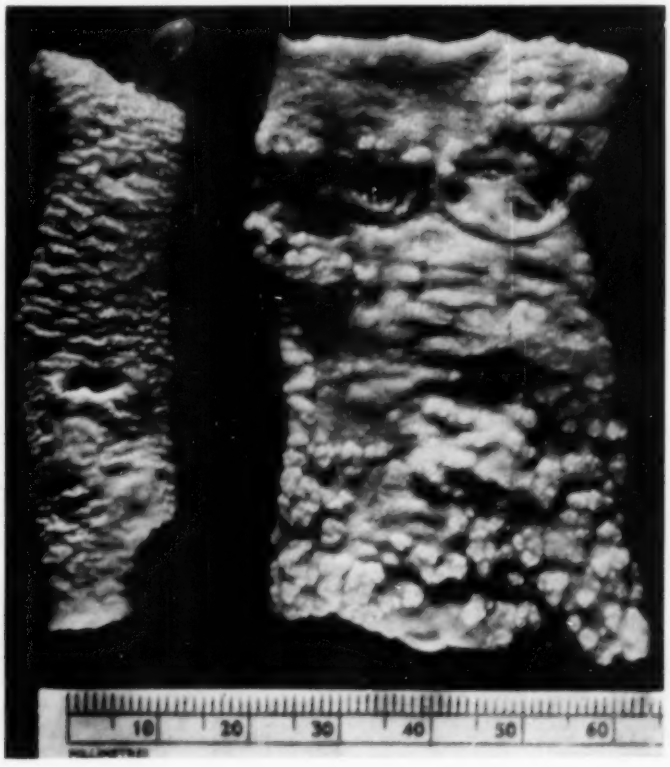

Fig. 3—Carotid artery (left) and jugular vein (right) from heifer showing extensive subendocardial calcification.

Small Arteries (Kidneys, Musculature).—These showed arteriosclerotic changes with thickening of the intima. In some sections, hyaline changes of the arteries of the kidney were seen (fig. 8).

Fig. 5—Articular surface of joint of heifer showing ulceration and degeneration.

Veins (Posterior Vena Cava).—There was a deposition of calcium mainly in the intima, but small amounts also in the media, with fragmentation and destruction of elastic tissues in the areas so affected. In some of the large veins, in addition to calcium deposits in the media, either isolated or in connection with small processes in the intima, there were areas of degeneration with surrounding inflammatory reaction. Apparently, subsequent collagen deposition and scarring resulted in these areas.

Lung.—The alveolar walls in certain areas were greatly thickened through accumulation of collagen and deposition of material which had the tinctorial characteristics of calcium. Capillaries were present in the thickened walls, and there was a tendency for calcium to be deposited on the alveolar sides of the wall.

It appeared that, initially, there had been a deposition of rather edematous-looking reticular tissue padding the alveolar wall, subsequently followed by deposition of collagen and calcification. Repetition of this process had resulted in greatly thickened calcified alveolar septums. Stages of this process are shown (fig. 9 and 10). Mucin was not found in these areas. Emphysematous areas were frequently present.

Concentric thickening of arteries and veins with hyalinization of the walls and, in some sections, obliterating endarteritis was seen.

Some sections showed the lesion to be so advanced that reconstruction of the morphogenesis was impossible.

Fig. 6—Section of endocardium of the heifer showing subendocardial calcification. Hematoxylin and eosin. x 43.

Kidneys.—In some cases, there were calcific deposits in the renal arteries and in the interlobular arteries where the intima, and sometimes parts of the media, were involved. No distinct changes were seen in the glomerular vessels but, occasionally, a protein-like material was present in Bowman's capsular space.

The proximal convoluted tubules showed hyaline droplet degeneration of the epithelium and contained casts which, in parts, appeared to be composed of a calcium-like substance, especially at, or close to, the corticomedullary junction. In material received from Buenos Aires, the tubular epithelial cells were markedly degenerated in aspect, despite the fact that fixing was thorough and made within 30 minutes after death of the animal.

Discrete calcium deposits were sometimes seen in the kidney parenchyma while in other sections an interstitial round-cell infiltration along with some cortical scarring, presumably arteriosclerotic in nature, was present. There were also foci of round cells in the corticomedullary region.

Bladder and Ureters.—No remarkable histopathological changes were observed.

Spleen.—There was a considerable amount of hemosiderin in the splenic pulp, also fibrosis, congestion, and calcium deposits in the capsule and in some trabeculae.

Lymph Nodes.—Except for widening of some sinusoids and an occasional eosinophilia, no remarkable histopathological changes were seen.

Abomasum.—There was deposition of

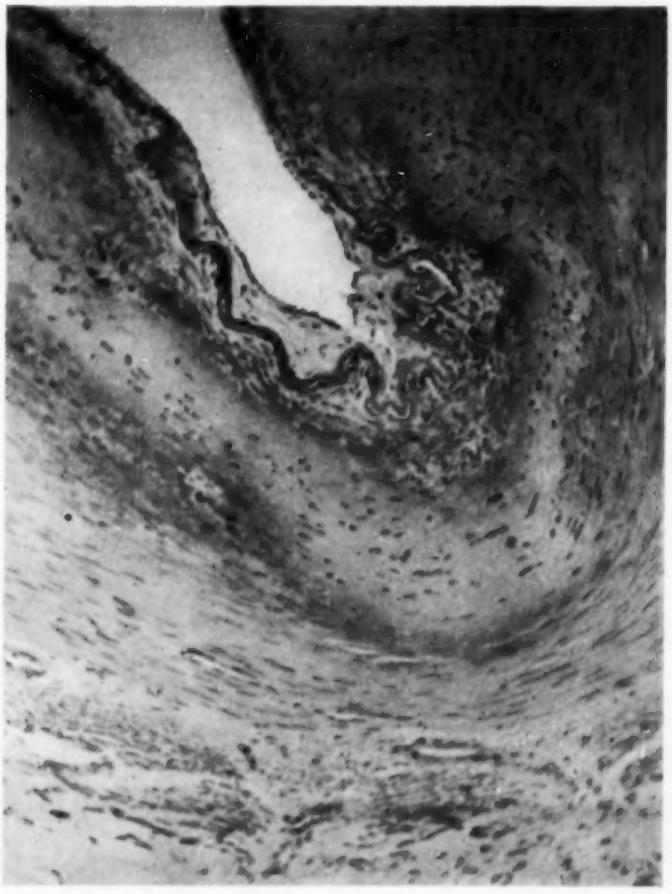

Fig. 7—Section of medium-sized artery of heifer showing calcification of media and reduplication of internal elastic layer. Hematoxylin and eosin. x 150.

calcium in the interstitial tissue of the mucosa in the fundus region, and also degeneration, necrosis, and calcification of mucosal epithelium in a few isolated areas. In one section, there was also an indication of calcium deposition in the muscular coat.

Small Intestines.—Hemosiderin was demonstrated in the subepithelial tissues.

Liver.—Glisson's capsule was greatly thickened and some areas showed deposition of calcium along with a mononuclear cellular infiltrate.

A chronic venous congestion was seen with focal increase of polymorphonuclear leukocytes, sometimes coinciding with areas of severe congestion. There was some increase of periportal fibrous tissue and lymphocytic infiltration of the portal triads. Bile pigment was present in some liver cells and there was hemosiderin in the Kupffer's cells.

Bone.—The bone appeared normal, histologically.

Skeletal Muscle.—Areas of calcification were observed, presumably occurring in association with small arteries.

Tendons.—There were calcific deposits, especially in fibrocartilaginous areas, such as near tendon insertions.

Diaphragm.—Some chronic inflammation with interstitial infiltrate and calcific deposits, surrounded by foreign body giant cells, was seen, especially in the fibrous part.

Endocrine Glands.—No histopathological changes were seen in the thyroid, pituitary, or adrenal glands.

Ovary.—No remarkable histopathological changes were seen.

Uterus.—No histopathological changes were noticed except calcium deposits in the arteries.

Skin.—No specific histopathological changes were seen.

BIOCHEMICAL FINDINGS

In animals affected with Manchester wasting disease, there appears to be an increase in the calcium content of the circulating blood (table 1, figures quoted from Arnold and Fincham [1]).

Comparison of Blood Calcium and Phosphorus Figures from Cows Affected with Manchester Wasting Disease and Normal

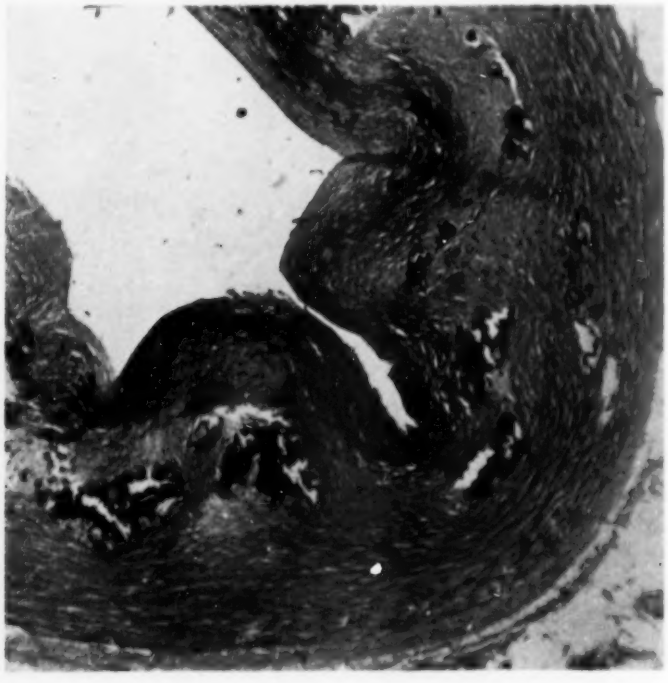

Fig. 8—Section of small artery of heifer showing calcification of subendothelium and media. Hematoxylin and eosin. x 43.

TABLE 1—Calcium Content of Circulating Blood of Animals Affected with Manchester Wasting Disease

	No. animals	No. herds	Serum calcium per 100 cc. of blood (mg.)	Serum phosphorus per 100 cc. of blood (mg.)
Cows affected with Manchester wasting disease	5	3	12.3	3.3
Clinically normal cows from "red earth" grazings	85	7	12.2	3.1
Normal cows from plains	40	3	11.6	3.0

These figures are considered more comparable than those quoted by Arnold in a later note[10] because of a change during that period in laboratory technique for the estimation of calcium. The above figures are directly comparable as a single technique was used throughout.

Animals from Similar Areas and from Lowland Grazings.—Chemical analysis of calcified material from an aortic plaque gave figures of 45.6 per cent of calcium oxide and 42.0 per cent of phosphoric anhydride which would correspond to an approximate composition of calcium phosphate ($CA_3[PO_4]_2$), 84 per cent and calcium carbonate ($CaCO_3$), 16 per cent.

Foreign Material.—Through the courtesy of Dr. Rosenbusch of Buenos Aires,[5] Dr. Mucciolo of Sao Paulo,[7] and Dr. Willers of Hawaii,[14] material of animals suffering from enteque and naalehu diseases was made available to us, and on visits to South America by one of the authors there was opportunity to perform necropsies and to collect material. Histological examination of these tissues revealed a picture similar to that in the Jamaican animals, except that in the material from Hawaii there were more severe histopathological changes in the heart than we had observed in the Jamaican specimens. Here the pres-

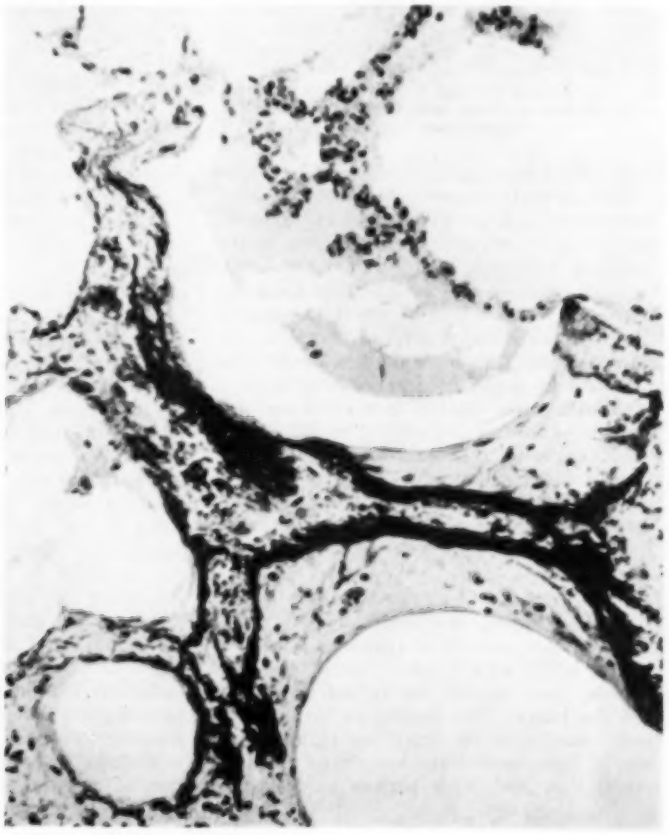

Fig. 9—Section of lung of heifer showing thickened alveolar septums with calcification and collagenization. x 260.

Fig. 10—Section of lung of heifer showing thickened alveolar septums with calcification and collagenization. x 173.

ence of calcium deposits had given rise to a foreign body reaction, while the subendocardial muscle fibers, which depend partly upon diffusion through the endocardium for their supply of nutriments, showed signs of anoxia and degeneration, apparently as a result of the thickening of the overlaying endocardium. Fibrous septums inside the myocardium were also found to be greatly thickened and to show calcific deposits. Aside from this one difference, which is considered to be due to these sections representing a more advanced stage than had our previous Jamaican ones, the material from all four sources showed an identical disease process.

DISCUSSION

The animals examined presented an emaciated appearance and showed a deposition of calcium salt in various soft tissues of the body, with a predilection for blood vessels, particularly the larger ones, and for the lungs. The lesions in the arteries and veins, with the resulting rigidity and loss of tone, must have interfered with the circulation and with proper oxygenation of the tissues. In advanced cases, the lungs suffered severely, presumably with loss of elasticity and severe impairment of respiratory movements. The greatly thickened alveolar walls must have impeded gaseous exchange. It is probable that such changes in arteries and lungs were factors in the development of hypertrophy of the right side of the heart and chronic venous congestion of the liver.

The hemosiderosis, as observed in the spleen and to a small extent in the liver and the gut, is not fully explained. There is little evidence that this might be correlated with breakdown of red blood cells. In some animals petechiae were noticed, probably as a result of local anoxia with damage to capillary walls causing hemorrhage.

The underlying cause of the pathological calcification can not be clarified from the morphology and will require additional experimental studies. It does appear, however, that the underlying disease process is a pathological calcification with certain preference for the organs discussed, rather than specifically a disease of blood vessels or any organ system of the body.

In the literature, pathological calcification is commonly classified in certain subgroups although it is not possible to make a clear distinction in all cases.

Dystrophic calcification is the deposition of lime salts in dead or degenerating tissues and is the most frequent type of pathological calcification. It occurs in extremely varied locations, common sites being in areas of tuberculous necrosis, in blood vessels in arteriosclerosis, in areas of fatty degeneration and necrosis, in degenerating thyroid tissue, and in degenerating tumors. Dystrophic calcification is not dependent upon an increase in the amount of calcium in the blood but rather appears to be dependent upon local microchemical reactions in the tissues. Local alkalinity in comparison with adjacent undamaged tissue appears to be the important factor initiating the precipitation of calcium in degenerating and dead tissues.

We have not observed any areas of necrosis or degeneration which might lead us to believe that such processes preceded the pathological calcification in Manchester wasting disease.

Metastatic calcification is the precipitation of calcium salts in tissues previously

normal but with a tendency toward alkalinity. This disease process in man has been excellently reviewed by Mulligan,[11] who states that bone disease, primary neoplasia of the parathyroid and hypervitaminosis D, alone, or in combination, have been incriminated as causative factors. Mulligan's review includes an evaluation of the experimental work done in animals in which parathyroid extract, vitamin D, and diets supplemented with various minerals have been employed to produce metastatic calcification. Another factor in the production of this calcification is retention of phosphate in the blood following damage to renal parenchyma as a result of a variety of kidney diseases.

Supersaturation of blood serum with calcium and phosphate ions, reduction of the concentration of hydrogen ions in the tissues, phosphatase activity, and tissue injury due to the initial calcific changes are other factors to be considered.

The histopathological and chemical findings in Manchester wasting disease support the hypothesis that the disease process involved is essentially a form of metastatic calcification. The possibility that a pathological parathyroid is operative in the present case is remote, in our opinion, as there appears to be no rarefaction of bone and the distribution of the condition according to breed, sex, or age, and its association with particular localities, appears to indicate that the cause is not hormonal.

Osteoporosis and massive deposition of calcium salts occurring in the soft tissues, including the kidneys, has been observed in man,[12] sheep,[13] and rats[14] following massive overdoses of irradiated ergosterol. The pathological changes in general are similar to those of Manchester wasting disease with the exception of the severity of the renal lesions, which we believe may be accounted for by the different pH of the urine in the different species. While the syndrome of Manchester wasting disease, as it occurs in nature, can not be directly attributed to an excess of vitamins of the D group, it shows many similarities to experimental or accidentally induced hypervitaminosis D.

Other authors, some of whom are listed by Hawkins *et al.*,[15] have attributed similar lesions in experimental work to various excesses or imbalances of the mineral constituents of their diets, and experimental and field data in Jamaica support the hypothesis that such factors are involved in the causation of Manchester wasting disease. While the precise etiological factor of this syndrome still awaits definition, experimental work to date (which will be reported in full in a future paper) suggests that excesses of calcium in the presence of a high calciferol intake, associated with an actual or conditioned deficiency of minor elements, may be implicated.

Summary and Conclusions

A detailed description of the histological changes found in Manchester wasting disease of cattle is given. The essential pathological process is considered to be a form of metastatic calcification. On the basis of a comparison of the symptoms and gross and microscopic pathology, the condition is considered to represent the same syndrome as those known as "enteque seco" in South America and "naalehu disease" in Hawaii.

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Abscesses of the Pharyngeal Region of Swine

Bacteriological Examination of Exudates

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THIS WORK was undertaken to determine whether or not Lancefield's group E Streptococcus [4] is regularly associated with a certain kind of abscess of the pharyngeal region of swine.

Stafseth and Clinton,[11] Snoeyenbos *et al.*,[10] and Collier[1,2] have associated this bacterium with this condition. Newsom[6] first described the gross lesions of this disease in swine in the United States. The abscesses range from less than 1 cm. to more than 10 cm. in diameter. The smaller lesions are embedded in the lymph nodes of the pharyngeal region. The exudate is characteristically nonodorous, distinctly greenish in color, and varies in consistency from creamy to glutinous. Superficially located abscesses are readily observable in the live animal, but deep lesions are difficult to detect and may be revealed only at slaughter.

The disease characterized above has been variously called "feeder boils," jowl abscesses, cervical abscesses, or strangles of hogs. A survey by Pickard,[9] including data from meat packing establishments located in various regions of the United States, showed condemnations of abscessed heads at slaughter to be as high as 7 per cent of the total hog kill in some plants. The operators of one establishment[7] of moderate slaughtering capacity in eastern Iowa estimated that they suffered an annual loss of at least $50,000 due to condemnations of swine heads because of this disease.

MATERIALS AND METHODS

Unopened abscesses were obtained from meat packing establishments for bacteriological examination. These specimens were selected by veterinarians of the federal meat inspection service and shipped in fresh chilled condition. The 310 specimens included in lot 1 (table 1) were taken from swine that were raised in eastern Iowa and western Illinois. The 128 abscesses included in lot 2 came (in about equal numbers) from northern Iowa, southern Minnesota, and Ohio. Lot 3 consisted of 54 specimens of exudate collected aseptically from the lesions of swine on farms located in various parts of Iowa.

Standard bacteriological methods were employed in the processes of initial isolation and pure culture study of the various kinds of bacteria encountered in the survey. Smears of the exudates were stained by Gram's method, and duplicates of 10 per cent of them were also stained by the acid-fast method. The exudates were streaked on bovine blood agar plates. The atypical appearing exudates were also streaked on sodium azide-crystal violet blood agar medium of the kind developed by Packer[8] for the isolation of streptococci. Duplicate blood agar plates, prepared from 10 per cent of all exudates, were incubated under anaerobic conditions. Ten per cent of all exudates were also inoculated on Sabouraud's medium.

A battery of carbohydrate mediums, found useful in the identification of the group E Streptococcus, included lactose, mannite, raffinose, salicin, sorbitol, trehalose, and inulin broths. Five per cent of all cultures that were identified as this group, on the basis of cultural and biochemical characteristics, were also subjected to serological grouping as a further means of identification. The serological method employed was a combination of the microprecipitin test developed by Lancefield[5] and the formamide extraction method developed by Fuller.[3] Part of the cultures thus identified were sent to Dr. Updyke[12] for confirmation.

RESULTS

No evidence of *Mycobacterium tuberculosis* was obtained on microscopic examination of the smears of the exudates that were stained by the acid-fast method. No fungi grew on the Sabouraud's medium. Inoculated mediums that were incubated anaerobically yielded no different kinds of bacterial growth than were obtained on duplicate mediums incubated aerobically.

On blood agar the group E Streptococcus grew slowly and showed little evidence of hemolysis after 24 hours of incubation; however, at 48 hours the isolated colonies typically measured 0.8 mm. in diameter and were surrounded by sharply defined circular zones of *beta*-type hemolysis measuring 1.5 to 2.0 mm. in diameter. Occasional strains of this organism induced no hemolysis on the blood agar. The nonhemolytic colonies possessed the high convex or globular appearance characteristic of all the hemolytic strains of this group encountered in this survey and, for that reason, were but little more difficult to

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detect on the blood agar. The blood agar cultures of occasional exudates were overgrown by Proteus sp. or crowded with various kinds of bacterial growth. The sodium azide-crystal violet blood agar medium was invaluable in isolating this group in these instances.

In beef infusion broth, the group E Streptococcus produced growth that was characterized by uniform turbidity, no pellicle formation, and a slight to moderate precipitate of cells. Chains of three to 16 gram-positive cocci were observed under the microscope in stained preparations from the broth medium. Acid production in carbohydrate mediums was usually apparent after 24 hours of incubation. This group characteristically produced acid but no gas in mannite, salicin, sorbitol, and trehalose broths, but not in lactose, raffinose, or inulin broths. Occasional strains were slow or negative in producing acid from sorbitol. Almost without exception, the Streptococcus produced a transitory reduction in litmus milk which was apparent after 16 to 24 hours but was reversed at 48 hours from the time of inoculation.

The group E Streptococcus was consistently isolated from all classes of specimens. There were 421 isolations of this microorganism from the 492 specimens examined for an overall incidence of 85.6 per cent. The breakdown in numbers of isolations of this group from various lots is shown (table 1). The higher percentage of pure cultures and total isolations of this organism from the exudates in lot 1, as compared to lot 2, was associated with a more careful selection of the specimens in the former lot to avoid excessive numbers of lesions in the advanced stage of development (*i.e.*, those with necrotic overlying skin). The highest incidence of pure cultures and total isolations of this organism, and the lowest incidence of other kinds of bacteria, were obtained in lot 3. These results would suggest that the incidence of this organism from the exudates of abscesses of the pharyngeal region of swine is a function of the selection of the specimens.

The following list includes the name and the over-all incidence of each of 11 kinds of bacteria, other than the group E Streptococcus, that were isolated from abscess exudates: *Corynebacterium pyogenes* (12.8%), *Pasteurella multocida* (5.89%), *Proteus ammoniae* (3.25%), *Streptococcus equisimilis* (1.42%), *Escherichia coli* (1.02%), *Streptococcus zooepidemicus* (0.81%), *Actinomyces bovis* (0.41%), *Spherophorus necrophorus* (0.41%), *Proteus vulgaris* (0.20%), *Salmonella typhimurium* (0.20%), and *Staphylococcus aureus* (0.20%).

TABLE 1—Incidence of Pure Cultures and Total Isolations of the Group E Streptococcus from the Exudates of Abscesses in the Pharyngeal Region of Swine

Lot (No.)	Sources of abscesses examined	Number of abscesses examined	Pure cultures of group E Streptococcus	Total isolations of group E Streptococcus
1	Packing plant A	310	217 (70.0%)	281 (90.6%)
2	Plants B, C, and D	128*	65 (50.8%)	88 (68.8%)
3	Various field cases	54	49 (90.7%)	52 (96.3%)
	Total:	492	331 (67.3%)	421 (85.6%)

* Seven abscesses in this group yielded no bacterial growth.

Discussion of Results

In order to be considered as a possible etiological agent of an infectious disease, a microorganism must be regularly associated with that particular condition. The group E Streptococcus, with an over-all incidence of 85.6 per cent, is the only bacterium, in this survey, that comes near to meeting this requirement. All remaining kinds of microorganisms obtained in the cultural survey may be excluded from consideration as possible etiological agents of the pharyngeal abscess disease of swine on the basis of infrequency of occurrence, or lack of known association with suppurative processes in swine, or lack of reputation as pathogens, or because of a combination of these reasons.

Summary and Conclusion

1) A *beta* hemolytic Streptococcus sp. belonging to Lancefield's serologic group E was isolated from 421 (85.6%) of 492 exudates from abscesses of the pharyngeal region of swine.

2) Eleven other kinds of microorganisms were isolated and identified from these same exudates. The incidences of these organisms ranged from 12.8 per cent to 0.20 per cent.

3) The group E Streptococcus is regularly associated with the exudate of a

certain kind of abscess that occurs in the tissues of the pharyngeal region of swine and, on that basis, is worthy of consideration as the etiological agent of that condition.

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The Effect of Octin on Baby Pigs Infected with Transmissible Gastroenteritis Virus

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THE EFFECT of transmissible gastroenteritis (TGE) upon the nutrition of baby pigs has been presented.[1,2] There is an abnormal loss of water and electrolytes by the pig when infected with this virus. This loss may be due to the increased propulsive motility of the intestines resulting in decreased opportunity for absorption. This is a report of an attempt, by the use of a drug, to reduce the propulsive motility of the gastrointestinal tract of pigs infected with TGE.

MATERIALS AND METHODS

A series of tests were made with octin.* One test evaluated its effects on the motility of isolated rabbit or swine intestinal sections; a second test evaluated its effects when administered orally to TGE-infected pigs less than 1 week of age; and a third test evaluated its effects when administered intramuscularly to TGE-infected pigs approximately 4 weeks old.

The technique as described by Reber[3] was used to determine the effect of octin when added to a bath surrounding an isolated intestinal section of rabbit or swine. This was done as a preliminary observation to the use of this drug *in vivo* to reduce propulsive motility of intestine in diarrheal conditions.

The same source of virus was used in the second and third tests. The virus was a strain originally recovered from a field outbreak in Illinois and had been passed through approximately 10 generations under laboratory conditions. Previous experience had shown that this strain of virus consistently produced symptoms of TGE in pigs 4 to 6 days of age. To prepare the exposure dose of virus, the gastrointestinal tract and contents of an exposed pig which had developed characteristic symptoms and lesions was finely ground with sterile sand and diluted with 9 times its volume of sterile physiological saline. All exposures were made *per os* by adding 1 ml. of this 10 per cent gastrointestinal tract suspension to the milk.

In the second test, the octin used was the commercially available product dissolved in water at the proportion of 2 gr. of octin to 50 ml. of water. Octin was administered to the pigs by adding appropriate amounts of this solution to the milk at feeding time as indicated in each experiment.

A total of 28 pigs between 4 and 6 days of age were used. These pigs were farrowed by apparently healthy sows and removed to individual modified Horsfall-Bauer units[4] at 3 to 5 days of age. On being confined in isolation units, they were offered 90 ml. of a mixture of 2 parts commercial evaporated milk and 1 part tap water[5] at 8:00 a.m., 12:00 m., and 5:00 p.m. All isolated pigs learned to drink this reconstituted milk within 12 hours of isolation.

The 28 pigs were separated into three groups designated A, B, and C (table 1). Two pigs in group A received neither octin nor virus, 2 received infectious material only, and 7 received

TABLE 1—Effect of Octin on Pigs Less than 1 Week of Age Infected with Transmissible Gastroenteritis Virus

No. pigs	Exposure	Treatment	Symptoms	Necropsy findings
		EXPERIMENT A		
2	None	None	None	None
2	1 cc. 10% GI tract suspension	None	+	+
7	1 cc. 10% GI tract suspension	1/12 gr. octin per feeding from exposure	+	+
		EXPERIMENT B		
2	None	None	None	None
2	1 cc. 10% GI tract suspension	None	+	+
6	1 cc. 10% GI tract suspension	1/25 gr. octin per feeding from exposure	+	+
		EXPERIMENT C		
2	None	None	None	None
2	1 cc. 10% GI tract suspension	None	+	+
3	1 cc. GI tract suspension	1/25 gr. octin per feeding beginning 24 hr. after exposure	+	+

+ Positive response to TGE infection; GI—gastrointestinal tract.

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* Each milliliter of octin contains 0.1 Gm. isomethептene, methylisо-octenylamine, hydrochloride, with 0.2 per cent paraben esters; Bilhuber-Knoll Corporation, Orange, N. J.

TABLE 2—Effects of Administration of Octin on Nutrient Balances of Noninfected Baby Pigs

	No octin*	Octin
No. of trials	12	2
Av. 24 hr. wt. gain (Gm.)	100	90
Range	53 to 163	81 to 99
Av. age (days)	29	28
Av. 24 hr. Na retained (mg.)	215	320
Range	69 to 346	303 to 337
Av. 24 hr. K retained (mg.)	488	538
Range	237 to 602	518 to 558
Av. 24 hr. N retained (mg.)	3422	3709
Range	2000 to 4272	3601 to 3817
Av. 24 hr. Na retained (%)	28	35
Av. 24 hr. K retained (%)	54	54
Av. 24 hr. N retained (%)	70	68

* Taken from Reber and Yusken.[2]

both infectious material and 1/12 grain of octin per feeding beginning at the time of exposure and continuing until death. Group B was treated in the same manner as group A except 6 pigs infected with TGE were given 1/25 grain of octin per feeding. Group C was treated in the same manner as group A except 3 TGE-infected pigs were given 1/25 grain of octin beginning 24 hours after exposure.

In the third test, older pigs were used. A previous report[2] described the use of 7 Hampshire pigs (4 noninfected and 3 TGE-infected) to determine the effects of a high dietary sodium level. Following the completion of that experiment, 2 noninfected pigs were given 1 ml. of octin intramuscularly daily. The feces, urine, vomitus, and excess feed were collected for a 96-hour period. All data (tables 2 and 3) are expressed as 24-hour averages.

Concurrent with the 3 TGE-infected pigs[2] were 3 TGE-infected litter mates which received octin. One milliliter of octin was administered intramuscularly at the time of feeding for four periods and at two hours after the time of feeding for six periods. Collections were made for 48-hour periods.

Results and Discussion

A final concentration of 0.28 mg. of octin per 100 ml. of Tyrode's muscle bath solution produced a considerable decrease in the amplitude of rhythmic contractions of isolated rabbit intestinal sections. However, this concentration had no effect upon the over-all length of the section, *i.e.*, it did not cause relaxation or contraction of the muscle section. The same concentration of octin added to a bath surrounding a section of a pig's intestine decreased the amplitude and produced relaxation (lengthening) of the section. Further tests revealed that the effect appeared to be proportional to the concentration of octin.

The *in vitro* swine intestinal section motility test indicated octin was effective in reducing the rhythmic motility and in producing relaxation of the intestine. This decrease in activity, when applied to the intact animal, theoretically should result in a decreased propulsive motility of the gastrointestinal tract. In turn, the decreased propulsive motility probably would enable the animal to make better use of nutrients which would be reflected in nutritional balances. This was the basis for using the drug on TGE-infected baby pigs.

The results of the second test using pigs 1 week of age or less are summarized (table 1). In all groups, the unexposed untreated pigs remained apparently healthy throughout the experiment and no significant lesions were found at necropsy. Signs of TGE, including vomition and severe diarrhea, developed in the other two groups within 24 hours after exposure. Necropsy revealed lesions of TGE including dehydration, hemorrhage into the fundic mucosa of the stomach, and atony of the small and large intestines. All exposed animals died between the fourth and eighth day postexposure.

The results of the third test in which octin was given intramuscularly to pigs

TABLE 3—Effects of Administration of Octin on Nutrient Balances of Baby Pigs Infected with Transmissible Gastroenteritis Virus

	No octin*	Octin**	Octin†
No. of trials	9	4	6
Av. 24 hr. wt. gain (Gm.)	31	61	−9
Range	−74 to 105	18 to 86	−74 to 29
Av. age (days)	26	24	28
Av. 24 hr. Na retained (mg.)	387	216	290
Range	52 to 648	106 to 362	202 to 368
Av. 24 hr. K retained (mg.)	341	−103	288
Range	148 to 506	−338 to 55	214 to 395
Av. 24 hr. N retained (mg.)	1774	849	1069
Range	−50 to 3955	−668 to 2555	111 to 2590
Av. 24 hr. Na retained (%)	51	36	46
Av. 24 hr. K retained (%)	42	−21	39
Av. 24 hr. N retained (%)	39	14	28

* Taken from Reber and Yusken;[2] ** octin given at time of feeding; † octin given 2 hr. after feeding.

approximately 4 weeks of age are summarized (tables 2 and 3). The administration of 1 ml. of octin to 1 of the 2 noninfected pigs caused vomiting on two separate occasions, once when given at feeding time and again when given 2 hours after feeding. It was observed that the noninfected pigs getting the octin were more active and excited than those not receiving it. The administration of octin to noninfected pigs did not affect their ability to retain sodium, potassium, or nitrogen (table 2).

The effects of octin upon the nutrient balances of TGE-infected pigs are presented (table 3). The 3 pigs infected with TGE given 1 ml. at feeding time were more inclined to vomit than the 3 infected pigs that did not receive octin. They also were more active, nervous and, for a short period after administration of octin, salivated excessively. The administration of octin to TGE-infected pigs prior to or at feeding time resulted in decreased retention of sodium, potassium, and nitrogen. Under these conditions, the pigs had a negative potassium balance. However, the administration of octin two hours after feeding time did not produce adverse symptoms nor adversely affect nutrient balances, nor was it beneficial in alleviating the conditions brought on by TGE infection.

SUMMARY

In vitro tests indicated octin added to a bath surrounding a swine intestinal section decreased the amplitude of rhythmic contractions and produced relaxation of the muscle. Observations were made on the effect of octin on the course of transmissible gastroenteritis in 16 pigs 4 to 6 days of age. Octin added to the milk at feeding time at the rate of 1/25 grain and 1/12 grain had no significant effect on the course of the disease under the conditions of these experiments. Octin was administered intramuscularly to pigs approximately 4 weeks of age infected with transmissible gastroenteritis virus. The administration of octin two hours after feeding had no effect upon the retention of sodium, potassium, or nitrogen. The administration of octin to TGE-infected pigs at feeding time produced vomiting and decreased their retention of sodium, potassium, and nitrogen. Octin does not appear to have therapeutic value in the treatment of pigs infected with TGE.

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A Survey of Tumors Occurring in Cattle, Sheep, and Swine

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THE NEED for surveys and detailed studies of the tumors occurring in domesticated food-producing animals in the United States has been more clearly recognized since Feldman's excellent monograph[13] in 1932 modernized the terminology and emphasized the importance of various groups of naturally occurring neoplastic diseases in animals.

Progress in the United States in the study of tumors of food-producing animals in the last two decades has been represented primarily by the appearance of case reports,[2, 5, 9, 10, 14, 23–25, 39, 45] general articles,[8, 47] and publications on groups of specific kinds of tumors which were of special interest.[7, 11, 15–17, 26, 28–30, 43, 53] In other fields of animal tumor research which were discussed by Feldman, advancements are evident in the studies conducted on neoplastic diseases of small animal pets,[1, 4, 19, 22, 31–34, 36, 44, 46] laboratory animals,[6, 37, 51] horses,[40–42] and captive wild animals.[38, 50]

Research workers in foreign countries have made limited studies of the occurrence of neoplastic diseases of food-producing animals in different locales of the world.[3, 18, 27] Their publications on specific kinds of tumors are too numerous to refer to although many of them represent outstanding contributions. References to such investigations in a recent paper[28] from this laboratory, on bovine uterine and lung tumors, illustrate the importance of evaluating and utilizing their observations in fields of tumor research in which we have neglected to obtain adequate basic information in the United States.

In 1950, veterinarians engaged in meat inspection pathology in the U. S. Bureau of Animal Industry became convinced that surveys and studies of neoplastic diseases conducted as an additional activity or project of their pathology laboratories could help to clarify the diagnosis of neoplasia in food-producing animals in this country.

Steiner and Bengston's excellent preliminary paper[47] indicates the role that the Federal Meat Inspection Service can play in aiding the study of neoplastic diseases in food-producing animals.

The Animal Disease Research Laboratory at Denver began a survey in 1953, to determine the relative occurrence of tumors in slaughtered animals at one stockyard. It was realized that the occurrence of tumors in one area of the country would not represent a completely accurate cross section of the problem but, nevertheless, it was an approach which could be attempted at that time.

The entire picture of neoplasia in food-producing animals can never be fully appreciated or understood by a study limited to tumors which are apparent at the time of slaughter. Nevertheless, few important types of tumors can escape detection in such a survey unless they consistently resulted in the death of young animals. Malignant lymphoma is recognized as a

TABLE 1—The Number and Type of Bovine Tumors Collected in 1953 and 1954

Type	1953	1954	Total
Epithelial Tumors			
Ocular squamous cell	722*	—	722
Adenocarcinoma of uterus	9	4	13
Metastatic adeno-carcinoma (probable uterine origin)	7	6	13
Carcinoma of the lung	3	—	3
Adenocarcinoma of the intestine	—	1	1
Ovary	3	4	7
Kidney	1	3	4
Gallbladder	3	7	10
Liver	5	3	8
Metastatic adenocarcinomas (not of uterine origin)	2	1	3
Adrenal cortical	8	15	23
Squamous cell carcinoma of skin	1	2	3
Metastatic squamous cell carcinoma (not primary ocular tumor)	1	—	1
Squamous cell carcinoma of vulva	—	1	1
Multiple papillomas of oral cavity	—	1	1
Nonepithelial Tumors			
Lymphoid	10	10	20
Schwann's cell	20	23	43
Adrenal medullary	2	3	5
Smooth muscle	6	2	8
Angioid	3	3	6
Fibrous connective tissue	5	2	7
Mesothelioma	1	1	2
Melanoma	1	1	2
Lipoma	—	1	1
Meningioma	—	1	1
Totals	813	95	908

* Partial collection for only the calendar year 1953; — no tumors collected.

From the Animal Disease and Parasite Research Branch Laboratory, Agricultural Research Service, U.S.D.A., Denver Federal Center, Denver, Colo. Dr. Davis is now with the Animal Disease Eradication Branch, ARS, U.S.D.A., Washington, D. C. and Dr. Monlux is with the School of Veterinary Medicine, Stillwater, Okla.

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TABLE 2—The Number and Type of Ovine Tumors Collected in 1953 and 1954

Type	1953	1954	Total
Epithelial Tumors			
Metastatic adenocarcinoma (primary site undetermined)	1	4	5
Adenocarcinoma of kidney	—	1	1
Adenocarcinoma of thyroid	1	—	1
Liver	3	5	8
Adrenal cortical	—	5	5
Squamous cell carcinoma of skin	1	1	2
Squamous cell carcinoma of vulva	—	1	1
Squamous cell carcinoma of eyelid	1	—	1
Nonepithelial Tumors			
Lymphoid	8	12	20
Myeloid	—	2	2
Adrenal medullary	3	6	9
Leiomyoma	—	1	1
Angioid	1	2	3
Fibrosarcoma	2	—	2
Chondrosarcomas	2	1	3
Lipoid	1	1	2
Totals	24	42	66

— No tumors collected.

comparatively frequent cause of death in calves and yet the tumor was repeatedly found in this survey in adult cattle. Our impression is that the majority of the tumors which result in the death of young animals of the bovine, ovine, and porcine species are of the lymphoid and myeloid types and that similar, but usually less anaplastic, tumors are also seen in older animals of these same species. There are a few tumors of younger animals which can be regarded as exceptions to this general rule due to their infrequent occurrence in older animals. One frequently brought to our attention is the malignant melanoma of young swine which may represent a serious problem. Many pigs in a herd may develop this malignant tumor and die before they reach market age. Feldman [13] makes reference to the observations of Caylor and Schlotthauer in one litter of purebred Duroc swine. Since no malignant melanomas and only three benign melanomas were detected in this survey, it can be concluded that melanomas

TABLE 3—The Number and Type of Porcine Tumors Collected in 1953 and 1954

Type	1953	1954	Total
Embryonal nephroma	4	9	13
Embryonic sarcoma of vagina	—	1	1
Lymphoid	3	6	9
Ganglioneuroma	1	—	1
Fibroma	—	1	1
Melanoma	3	—	3
Totals	11	17	28

— No tumors collected.

are not an important tumor in hogs coming to slaughter.

Trotter,[49] Joest,[20] and Sticker [48] did some of the more thorough early work on animal tumors prior to 1932. The 150 tumors described by Davis, Leeper, and Shelton [8] in 1933 had been sent to Feldman for diagnosis and were the major source of that type of specimen for his 1932 monograph.[13]

Collection of Data

A review of all cases of neoplastic diseases of food-producing animals in the files at the Denver Animal Disease Research Laboratory was conducted in 1951.[28] Most of these tumors were collected as a result of curiosity about the identity of a lesion or concern about the proper disposition of a carcass. To illustrate the inaccuracy of estimating the occurrence of tumors in reference to the submitting of routine meat inspection specimens to the laboratory, metastatic adenocarcinomas of probable uterine origin were the most frequently

TABLE 4—The Number of Bovine, Ovine, and Porcine Tumors (Excluding Bovine Ocular Tumors) Collected Each Month in the Two-Year Survey

	1953				1954			
	Bovine	Ovine	Porcine	Total	Bovine	Ovine	Porcine	Total
January	6	4	—	10	2	1	—	3
February	2	1	1	4	10**	—	1	11
March	7	1	2	10	12	3	1	16
April	5	3	2	10	6	3	1	10
May	4	1	1	6	7	—	2	9
June	1	1	—	2	3	8	3	14
July	8	1	1	10	7**	9	1	17
August	6*	2	—	8	8	5	—	13
September	7	5	1	13	6	3	1	10
October	10	2	—	12	7*	3	—	10
November	24**	1	1	26	16**	4	1	21
December	11	2	2	15	11*	3	6	20
Totals	91	24	11	126	95	42	17	154

— No tumors collected; * an ocular tumor was found in one animal in addition to the tumor included in this table; ** two recorded tumors found in one animal in each of these groups.

submitted carcinomas (even exceeding ocular carcinomas), and the common Schwann's cell tumors represented only a small fraction of the neoplasms examined.

During 1952, permission was secured from the Federal Meat Inspection Service for veterinarians to assist in conducting and evaluating detailed necropsies of animals with neoplastic disease at the Denver abattoirs.

TABLE 5—Total Number of Animals Slaughtered in the Calendar Years 1953 and 1954 at Denver's Federally Inspected Abattoirs

Species	1953	1954
Cattle	594,083	736,632
Calves	49,261	49,409
Sheep	563,435	548,417
Hogs	485,876	496,824
Goats	2	—

— No animals of this species were slaughtered.

In 1953 and 1954, all bovine, ovine, and porcine tumors diagnosed in the routine inspection of slaughtered animals were collected and, when possible, detailed necropsies were continued. Many bacterial and parasitic granulomas and a variety of other lesions were submitted because it was thought, on gross examination, that they might be neoplastic. As an example of such diagnostic problems, degenerated tapeworm cysts in the bovine heart must be differentiated from some types of cardiac Schwann's cell tumors. The monthly variation in the numbers of tumors collected during the calendar years of 1953 and 1954 is shown (table 4) and illustrates why such surveys should extend over complete calendar years. It is explained later in this article, and in a subsequent article,[27] why the collection of bovine ocular neoplasms was limited to a sampling in selected abattoirs only during the calendar year of 1953.

Breed, age, sex, physical condition, market value, productivity, and actual numbers of animals slaughtered, obviously would be important factors in the seasonal appearance of neoplasms in abattoirs. Seasonal variations in the type of cattle submitted for slaughter are perhaps slightly more exaggerated in Denver than in most livestock marketing centers. This is due, in part, to the many beef breeding herds maintained in the surrounding country and the location, or at least seasonal grazing, of at least one third of these in the mountains or high, inaccessible valleys. Since approximately 82 per cent of the cattle slaughtered in the survey were Herefords,[27] the majority of the bovine tumors was found in that breed.

The actual number of animals slaughtered during the survey (table 5) could not be used to explain the increased occurrence of the tumors in sheep in the summer of 1954 (table 4). Severe culling of mature breeding animals in regional drought areas could have been a major factor responsible for this increase as it resulted in the slaughter of many more animals in the "cancer age."

The 1,002 tumors collected in our two-year survey are classified for cattle (table 1), sheep (table 2), and swine (table 3). In the classifications in the tables, the groups were made as simple as possible so that the types encountered would be more easily comprehended. The groups listed will be discussed individually for each species.

The variety of tumors encountered in the survey makes possible only brief general observations on each group. No photomicrographs but some gross pictures are included. Remarks in the section on bovine Schwann's cell tumors and bovine lung and uterine carcinomas represent a supplement of further pertinent observations to two recent publications.[28, 29] Observations on bovine ocular squamous cell tumors will be reported separately[27] and will be mentioned only in the general discussion and evaluation of this survey. The collection of these ocular tumors was limited by necessity to a study of a large representative group over a 12-month interval because of their frequent occurrence.

BOVINE EPITHELIAL TUMORS

Ocular Squamous Cell Tumors.—The 722 squamous cell tumors represented eye tumors found in 532 cattle in three of the larger Denver abattoirs during 1953, whereas reported necropsy diagnoses of epithelioma of the eye were made in 3,079 cattle in all of the Denver abattoirs in 1953.[27] Of the 532 cattle, 52 had lesions in both eyes.

A diagnosis of squamous cell carcinoma of the eye was made in 471 of the 722 tumors, and the remainder was regarded as early squamous cell carcinomas, epidermal plaques, or epidermal papillomas. Of the 471 squamous cell carcinomas, 22 had metastasized.

Adenocarcinoma of the Uterus.—Our recent report[28] on 26 cases of adenocarcinoma of the bo-

vine uterus included the 13 uterine carcinomas found in this survey. Lymph node metastases were found in all 13 cases; 12 also had recognizable lung metastases and 8 had peritoneal metastases. Of the 13 cows, 7 were Herefords; the breed was not recorded for the other 6.

Tissues from several additional adenocarcinomas of the uterus with metastases have been examined since the completion of the survey. How great a factor sterility may have been in influencing owners to send cattle affected with adenocarcinoma of the uterus to the market is not known but there are references [28] to sterility being the reason for slaughter of affected animals. One animal with a metastatic uterine carcinoma died 40 days after a dystocia had been reported.[28] A recent specimen of the entire bovine genital tract submitted from Sioux City, Iowa, is our only example of a fetus in one cornu with a typical adenocarcinoma in the midportion of the opposite horn. The cow was 10 years old and in poor condition but the 2- to 3-month fetus appeared normal. There were numerous metastases to the lungs and bronchial, sublumbar, and internal iliac lymph nodes.

Metastatic Adenocarcinoma (Probable Uterine Origin).—These 13 carcinomas were included in a report [28] of 62 cases of probable uterine carcinoma. Lymph node and lung metastases were found in all 13; 4 also had peritoneal metastases. The 13 cows included 9 Herefords, 1 Holstein-Friesian, and the breed was not recorded for the other 3.

Carcinoma of the Lung.—Three carcinomas of the lung were previously reported.[28] Two had been diagnosed as undifferentiated cell carcinomas and one as a mixed cell carcinoma. Two had bronchial, mediastinal, and cervical lymph node metastases and one had bronchial and mediastinal lymph node metastases. The breed of 2 was not recorded but 1 was a Hereford.

Single cases of a mixed cell carcinoma and an adenoma of the lung have been found since the survey. The carcinoma was in a 3-year-old cow and had a multiple focal distribution to the lungs and bronchial, mediastinal, and cervical lymph node metastases. The adenoma of the lung is of particular interest because it was found in an old cow in poor physical condition, whereas the three previously reported cases [28] had been in young animals in good condition. The lungs from the cow did not noticeably collapse at slaughter; they contained multiple, and often confluent, masses of a yellow, fibrous, partially calcified, neoplastic tissue which replaced much of the lung parenchyma. Multiple sections of this tumor were identical in microscopic appearance to the other adenomas except for large areas of necrosis, calcification, and other degenerative changes believed related to aging. An especially detailed examination of the bronchial and mediastinal lymph nodes was made at the necropsy but no evidence of early metastases was found.

Adenocarcinoma of the Intestine.—Adenocarcinomas of the bovine gastrointestinal tract are rare; only one with a definite primary site was found. When they occur, a small primary site, such as was encountered in our single case (fig. 1), may be overlooked. In microscopic examination of this primary growth, we were impressed by the anaplasia of the neoplastic cells, their invasiveness, and the many lymphatics which were plugged with tumor cells. In both the intestinal and the metastatic lymph node lesions, the neoplastic cells sometimes formed small glandular lumina or distinct acini with a low columnar epithelium. However, more often, the tumor was represented by closely packed clumps or strands of polyhedral to spindle-shaped cells. The cellular masses had some resemblance to the argentaffin carcinomas of the intestine described in man but no silver-stainable granules could be demonstrated in them. Variations in size of nuclei and many mitotic figures were additional indications of the malignancy of the tumor.

Among the three metastatic adenocarcinomas collected in this survey, whose primary site was not apparent and which were obviously not metastatic uterine carcinomas, two were thought to represent metastases of a primary adenocarcinoma of the intestinal tract. No primary site could be identified so this conclusion was based on the appearance and location of the metastatic lesions. It is noteworthy that these two cases, and the one discussed above, were all quite different in microscopic appearance.

One of these metastatic adenocarcinomas was found in a 7-year-old cow, with lesions in the liver, mediastinal and renal lymph nodes, and on the serosa of the gallbladder, spleen, rumen, omentum, peritoneum, broad ligament, and mesenteries. Histologically, the epithelium of the neoplastic glandular structures was a columnar type and many of these epithelial cells had basally placed nuclei. Occasional spherical mucous cells, which resembled goblet cells of the intestine, were present. The location and appearance of the metastases were compatible with a primary intestinal carcinoma.

The other probable metastatic intestinal carcinoma was found in an old Hereford cow. Lesions seen at necropsy were a large pelvic growth (32 x 30 x 18 cm.) which was adhered to the broad ligament and horns of the uterus and many tumor implants (up to 5 cm.) scattered on the abdominal serosa. Histologically, these were masses of a very anaplastic epithelial tumor. The neoplastic cells formed occasional glandular structures and were also identifiable as spherical, mucus-containing cells with their nuclei located at one side (signet ring cells).

Illustrated Case

Figure 1.—Adenocarcinoma of the intestine with lymph node metastases in a 9-year-old cow is shown (case D 2855).

Necropsy.—The primary tumor (2 x 2 x 1 cm.) was found in the jejunum and had

an ulcerating, partially necrotic and hemorrhagic surface. On section, this growth was represented by an irregular, infiltrating, predominantly white to grey mass which extended to, but not through, the serosa. Metastases were found as soft, grey, not visibly encapsulated lesions in the portal and some of the mesenteric and mediastinal lymph nodes and one internal iliac lymph node.

Fig. 1 — Adenocarcinoma of the jejunum in a 9-year-old cow. The tumefied ulceration of the mucosa shown in the center of the figure extended almost through the wall of the intestine (case D 2855).

Ovarian Tumors.—The group of seven epithelial ovarian tumors consisted of six granulosa cell tumors in which metastases were not seen and one cystadenocarcinoma in a 10-year-old cow, with metastases on the peritoneum and in the internal iliac, renal, sublumbar, mesenteric, mediastinal, and posterior cervical lymph nodes.

Granulosa cell tumors frequently appear in younger cows. The ages of the 6 in this survey were: 2 years, 2; 3 years, 2; 7 years, 1; and 1 was described as an old cow (in our entire station collection of 25 bovine granulosa cell tumors, over half were found in animals under 4 years of age). The six granulosa cell tumors were unilateral except in the old cow (a small tumor, only 5 mm. in diameter, was present in a relatively intact ovary; the opposite ovary was replaced by a massive tumor with dimensions of 40 x 18 x 15 cm.). Of the 6 animals affected, 4 were Herefords, 1 an Aberdeen Angus, and the breed of 1 was not recorded. Moderate endometrial hyperplasia but no cystic change was seen histologically in the uterus of the 7-year-old Aberdeen Angus cow, while the uterine mucosa of the other cows was considered to be essentially normal.

In 4 of the 6 animals with granulosa cell tumors of the ovary, the regional lymph nodes (internal iliac and sublumbar) were greatly enlarged, hyperplastic, and of a rather uniform deep red color. On histological examination of these nodes, there were masses of an iron-staining pigment in phagocytes in the medullary and cortical sinuses and, in 2 animals, a variable secondary inflammatory reaction, including formation of giant cells. Such lymph node lesions must be differentiated from metastases by histological examination. In our paper on uterine tumors,[20] we mentioned the absence of associated granulosa cell or other ovarian tumors, also the similarities of ovarian and uterine tumors in respect to routes of metastasis across the peritoneum or by the lymphatics.

In the present survey, one of the tumors listed as a metastatic adenocarcinoma was considered most apt to be a primary cystadenocarcinoma of either the ovary or fallopian tubes. Unfortunately, the genital tract had been discarded and the only lesions seen at necropsy were multiple peritoneal implants.

Illustrated Cases

Figures 2 and 3.—A granulosa cell tumor of the ovary of a 3-year-old Hereford is shown (case D 7203).

Necropsy.—An oval, solid, encapsulated, smooth surfaced growth (16 x 15 x 18 cm.) weighing 2 lb. 13 oz. replaced the right ovary (fig. 2). Large veins were visible on the surface of the tumor and, on section, many vascular spaces were interspersed in the neoplastic tissue. Small cystic spaces filled with a pale yellow fluid and yellow necrotic areas were also scattered among the lobulated, solid, greyish, tumor masses which made up the bulk of the growth (fig. 3). No remnant of the right ovary was found and the fallopian tube could be traced to a point where its proximal end was adhered to the capsule of the tumor. The left ovary appeared normal and there was no visible thickening or cystic change

in the endometrium of the uterus. No metastases were found.

Kidney Tumors.—Of our four epithelial tumors of the kidney, two were embryonal nephromas and two were adenocarcinomas. Although embryonal nephromas are actually embryonic tumors and contain undifferentiated mesenchymal elements, they will be discussed with the epithelial tumors in our simplified classification because of their neoplastic epithelial structures which make them closely related to the other kidney tumors.

One adenocarcinoma (fig. 4) was not only bilateral with multiple lesions in each kidney but was malignant in appearance and had liver and lung metastases. The cow was old and of mixed breed. No age or breed was recorded for the second animal. This tumor was massive (17 x 13 x 12 cm.) and thinly encapsulated, and had replaced several lobes of one kidney at its anterior extremity. No metastases were found although the regional lymph nodes were hyperplastic.

One of the embryonal nephromas was found in a 2-year-old Hereford steer (fig. 5) and the other in an old cow whose breed was not recorded.

Grossly and microscopically, both resembled the similar and much more common tumor found in swine. The tumor in the old cow had histological features which may have been related to aging and metaplasia; namely, many large epithelial-lined cystic spaces, nests of squamous epithelial cells which often had central epithelial pearls, and masses of cartilage interspersed throughout the tumor. Metastases were not observed in either of the 2 animals, but recently in a 2-year-old cow an

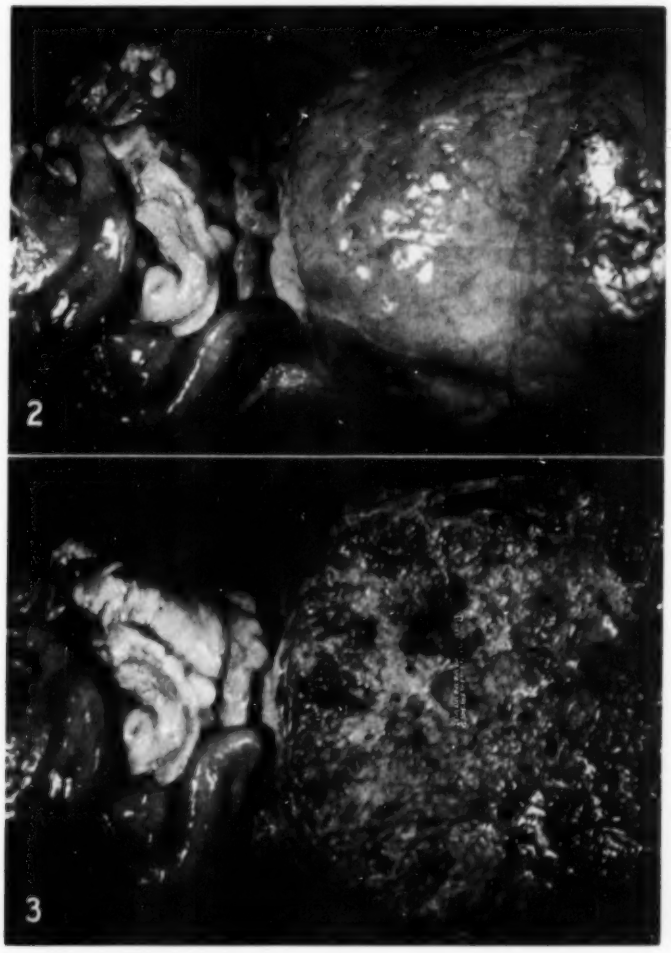


Fig. 2 and 3—An encapsulated granulosa cell tumor of the ovary in a 3-year-old Hereford cow; at the right before (fig. 2) and after (fig. 3) being bisected. The adhered fallopian tube and portions of the uterine cornua are included on the left (case D 2703).

embryonal nephroma was examined which was composed predominantly of undifferentiated mesenchymal cells and had lung, bronchial, and renal lymph node metastases. Feldman mentioned one embryonal nephroma, with lung and lymph node metastases, among the 250 cattle tumors discussed [12] and this same case was later reported by Davis *et al.*[5]

Illustrated Cases

Figure 4.—Adenocarcinoma of the kidney with liver and lung metastases in an old cow of mixed breed is shown (case D 3110).

Necropsy.—Both kidneys were tumefied and the distribution of neoplastic masses in each was similar; the cortex and medulla contained firm, grey to yellow, predominantly oval, thinly encapsulated or nonencapsulated growths varying from 2 to 8 cm. in diameter. The largest tumor in each kidney (8 and 7 cm., respectively, in their greatest diameter) was found approximately in the same location in each kidney, replacing much of the regional cortex and medulla and protruding into the hilus. In each of these larger growths, there were large hemorrhagic and necrotic central masses which were either very small or absent in the other kidney lesions (fig. 4, kidney incised to show several tumor masses and the one 8 cm. in its greatest diameter). Some of the tumors in each kidney had penetrated through the capsules and one had invaded the aorta and

Fig. 4 — Cut surface of an adenocarcinoma of the kidney in an old cow. The smaller tumors at the upper left and lower right may have been initiated by intrarenal metastases from the large (8 cm.) growth (arrows) (case D 3110).

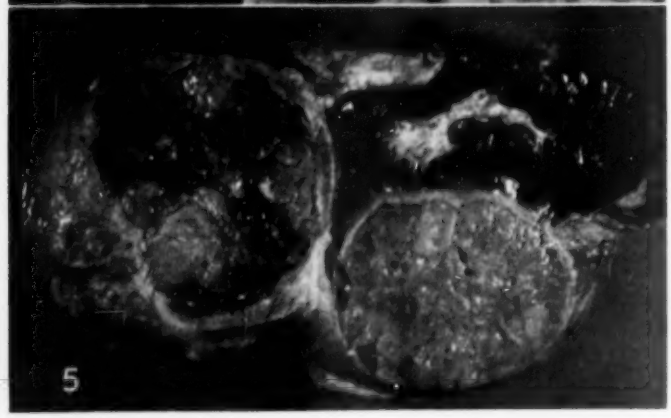


Fig. 5—Cut surface of an embryonal nephroma of the kidney in a 2-year-old Hereford steer. Intact lobes of the kidney are seen at the upper right (case D 2341).

formed nodular masses on its intima. The regional lymph nodes were enlarged, swollen, and hyperplastic and one renal lymph node contained a small necrotic center. Tumor metastases were not found in any of the lymph nodes. A large, encapsulated mass of neoplastic tissue (10 cm. in diameter), which was similar to those seen in the kidney, was present in the dorsal lobe of the liver. Tumor nodules (5–20 mm., dia.) were also found directly beneath the pleura in scattered locations in both lungs.

Figure 5.—Embryonal nephroma of the kidney in a 2-year-old Hereford steer is shown (case D 2341).

Necropsy.—An irregularly lobulated, encapsulated tumor (20 x 11 x 11 cm.) extended over and compressed about two thirds of the free surface of one kidney. On section, the tumor was firm, grey, and contained many small cysts. Many softer and pinkish lobules of tissue in the growth were believed to represent neoplastic tissue undergoing necrosis. Fibrous trabeculae almost completely divided the tumor into six separate lobules, and within each of these lobules, there were many small nonencapsulated lobular masses.

Gallbladder Tumors.—The ten epithelial gallbladder tumors in this survey were an interesting group and a report is being prepared for publication. Of the ten tumors, nine were diagnosed as papillary cystadenomas and one as a papillary adenocarcinoma. They were found predominantly in young animals. Their ages were: 2 years, 2; 3 years, 3; 4, 5, and 6 years, 1 each; and 2 were old Hereford bulls. It is noteworthy that 2 were bulls, 6 were steers, and only 2 were cows. Of the 10 affected cattle, 7 were Herefords and the breed was not recorded for the others. Of the nine other cases diagnosed at this laboratory, six were in cattle under 3 years of age and 5 of the 9 were steers.

The younger affected animals were in good or excellent condition at the time of slaughter. The tumors were remarkably similar in gross and microscopic appearance and were detected at necropsy by palpation of the distended gallbladder. The tumors were represented by a solitary growth except in a 2-year-old heifer which had two independent tumors in the fundic portion of the organ.

In only 2 of the 10 animals were the tumors attached to the fundic end of the gallbladder. The basal area of attachments varied from 1 to 4 cm. in their greatest diameter and were always represented by a red, rugose, epithelial-covered mass. From these basal areas, papillary folds and fronds extended for distances varying from 2 to 7 cm., often into the neck of the gallbladder. A simple columnar epithelium covered the papillary projections and was continuous with underlying tubules and glands of the tumor and the adjacent gallbladder mucosa. Numerous cystic glands and tubules were always present in the growths.

Our single case of adenocarcinoma, in a 6-year-old Hereford cow, was one of those in which the point of origin was not in the fundic end; it was located midway between the neck and fundic end. The growth had invaded the wall of the gallbladder into the adjacent liver. The portal lymph node was also tumefied; no other metastases were found.

In a 4-year-old steer, small black calculi were found in the fundic end and represented the only gallstones recovered from our ten cases. The tumor in this gallbladder was present about equal distance between the neck and the fundic end.

Liver Tumors.—Of the eight primary epithelial tumors of the liver, seven were liver cell carcinomas (malignant hepatoma, hepatocellular carcinoma) and one was a bile duct carcinoma (malignant cholangioma, cholangiocellular carcinoma). Metastases or an associated hepatic cirrhosis were not observed in any of these animals. Although we have specimens of mixed bile duct and liver cell tumors (hepatobiliary carcinomas) and both kinds of adenomas in our files, these types of tumors were not found in this survey. In studying epithelial liver tumors, the classification recently advocated by Edmondson and Steiner[12] in their study of human tumors was followed. All of our eight liver cell carcinomas would be grouped in their adult or mature cell type (grade II) of a liver cell carcinoma with a trabecular structure. Recognition of a trabecular pattern in at least a portion of each growth is important in diagnosing liver cell tumors because the trabecular growth represents an attempt by the neoplasm to reproduce the structure of the normal liver.

The bile duct carcinoma was a solitary, massive, greyish tumor (25 cm., dia.) in the dorsal lobe of the liver of an old cow. Only portions of the growth differentiated microscopically from solid epithelial masses into either small or large acinar structures.

The liver cell tumors often had different colors, sometimes even when present as multiple growths in the same liver. The concept of Edmondson and Steiner, that (1) the more mature liver cell types have a light brown appearance; (2) the more immature ones are grey-white; (3) bile formation or biliary obstruction results in varying shades of yellow and green; and (4) necrosis and hemorrhage is represented by dull brown to red-purple colors, seemed reasonably accurate in evaluating the gross and microscopic appearance of our collection of hepatic tumors. Lesions from 3 of the animals were varying shades of green, 1 had a solitary yellow lesion, 1 a single grey lesion, and the remainder had lesions of mixed orange, yellow, red, and purplish black.

Four of the liver cell carcinomas were represented by solitary lesions and all of these were at

least 20 cm. in their greatest diameter. The multiple tumors in the remainder were smaller but did produce a great enlargement of the respective livers. One old Hereford cow that had multiple, very fibrous, nodular, raised lesions in contrast to the softer and more parenchymatous tissue of the other liver cell tumors was so thin and cachectic that she was killed subject to the findings of the necropsy. All of the 7 cattle afflicted with liver cell carcinoma were females; their ages were: old, 4; 7 years, 2; and 6 years, 1. Four of the 7 were Herefords and the breed was not recorded for the remainder.

Metastatic Adenocarcinomas (Not of Uterine Origin).—Of the three metastatic adenocarcinomas, two were considered to have their primary site in the gastrointestinal tract and the third in the ovary or fallopian tube. They are discussed in the sections on adenocarcinoma of the intestine and ovarian tumors.

Adrenal Cortical Tumors.—The epithelial cortical tumors represented one of the most frequently diagnosed primary type of neoplasm of an individual bovine organ. All of the 23 tumors were remarkably similar in microscopic appearance.

[Some histologists are hesitant to call the adrenal cortex an epithelial structure although the appearance of individual cells may justify such a designation. Karsner[27] defended the classification of human adrenal cortical tumors as adenomas and carcinomas, even though acinar structures are absent, when he stated that they have origin in the epithelium of a true gland. His criteria for separating adenomas from carcinomas were invasiveness and evidence of metastases. Karsner believed that, in man, adrenal tumors occur more frequently in the female and that the majority of the cases represented by a hormonal phenomena were related to an adenoma producing excessive hormones during childhood and adolescence.]

The 23 adrenal cortical tumors in 186 neoplasms is a slightly smaller percentage than the report of Davis *et al.*[5] of 13 in 81 bovine tumors (bovine ocular tumors are excluded in this consideration in each survey). However, 15 of the 95 tumors examined in the second year of our survey were found to be of the cortical adrenal type. This is almost identical to the percentage recorded in the Davis study in some of the same Denver abattoirs. Feldman[7] regarded 9 of 13 adrenal cortical tumors in the Davis collection as carcinomas although none of the cases was metastatic. No metastases could be established in the 23 cases in this survey but we do have two adrenal carcinomas with lung metastases among 55 other adrenal cortical tumors in our files. Feldman[7] reported metastases in three of 12 adrenal carcinomas in his series, but did not mention how selective he was in making his collection or how many adrenal adenomas he observed.

In attempting to establish a basis for a diagnosis of adrenal cortical carcinoma in cattle, we concluded that the factors of invasiveness and actual size were most important in the absence of actual metastases. Otherwise, except for variable depositions of fibrous connective tissue and calcium and areas undergoing necrotic and cystic changes, the tumors were remarkably similar in appearance. Our views on these tumors are in agreement with Willis[35] when he states "no sharp separation of benign and malignant growths is possible; whether a large circumscribed but growing tumour shall be regarded as an unusually active 'adenoma' or as a 'carcinoma' which has not yet displayed invasiveness or metastasis, is a matter of personal preference."

When the actual sizes of the cortical tumors were summarized in our survey, they were consistently large; only four were under 5 cm. in their greatest diameter, nine were 5 to 10 cm., nine were 10 to 20 cm., and one in an old cow was 25 cm.

The second factor of invasiveness was more difficult to evaluate. All of the cortical tumors, except a few of the smaller growths, had a thick, fibrous capsule. As our study of these tumors progressed, it was recognized that if the tumor in its growth contacted rather firm, adjacent tissue (notably liver, kidney, or aorta) the capsule covering the contact surface of the neoplasm often was very thin. In the liver and kidney, fibrous adhesions formed on their involved peritoneal surfaces with little recognizable parenchymatous changes within the organs. Similar adhesions were seen in 2 animals where tumors had expanded to contact the aorta, and five other tumors were found adhered to and penetrating through the aorta. Small, lobular, thinly encapsulated neoplastic masses characteristically protruded into the lumen of the aorta (fig. 7). Sections of these five tumors did not show any convincing evidence of invasiveness of individual or small groups of neoplastic cells. We have concluded that pressure produces an atrophy of the aortic wall and eventually a thinly encapsulated outpouching of the tumor extends into the lumen of the aorta. These five tumors were 19, 18, 17, 16, and 10 cm. in their greatest diameter and would be included among our ten largest adrenal tumors. There would be justification for regarding the ten tumors as potential trouble makers and grossly as malignant tumors.

The penetration into the aorta rather than into the vena cava is of particular interest as it is the vena cava which is most commonly invaded by tumors in man. Obviously, different anatomical relationships must occur in reference to the expanding tumor in cattle. Of the ovine adrenal medullary tumors, two were found penetrating the vena cava and one penetrating the aorta. One bovine pheochromocytoma had penetrated into the aorta.

Of the 23 animals with cortical tumors, 18 were at least 6 years old; 11 of these were listed only as old animals. The age of the other 5 was: 3 years, 2; 2 years, 1; 5 years, 1; and not listed, 1. Ten were Herefords, 1 was of mixed breeding, and the breed was not listed for 12. Two were steers and 21 were females. A bilateral involvement was seen in 1 old cow. The smallest tumor found was

only 2 mm. in diameter and was an incidental finding in an animal which also had a large Schwann's cell tumor of the adrenal ganglion. Cortical nodules were seen in several animals including a few with nodules in the periadrenal fat. They were considered as hamartias rather than true tumors and will not be discussed in this paper.

No evidence of endocrine imbalance was detected in any of these animals, or in any cases in our file. Our attention has never been directed to adrenal tumors in calves. However, if age is a factor, a corollary might be drawn to the tumors in man, and one should be aware of possible excessive hormonal stimulation, if such a tumor is encountered.

Illustrated Cases

Figure 6.—Adrenal cortical adenoma in a 6-year-old cow is shown (case D 2923).

Necropsy—An irregularly oval, encapsulated growth (9 x 8 x 6 cm.) was attached to and had replaced a portion of one adrenal. On section, the tumor was divided into many poorly defined, firm, grey lobules of tissues which contained many tiny grains and a few larger masses of calcium. Recognizable cysts were not found in the growth.

Figure 7.—An adrenal cortical carcinoma in an old cow is shown (case D 2688).

Necropsy.—An oval encapsulated tumor (10 x 7 x 4 cm.) arose from one border of a partially intact adrenal gland. The tumor was adhered to the wall of the posterior aorta. A thinly encapsulated rounded projection from the tumor (about 1 cm., dia.) protruded through the wall of the posterior aorta. The adjacent portions of the aorta near the point of penetration were

Fig. 6—Cut surface of an adrenal cortical adenoma in a 6-year-old cow. An incised remnant of adrenal (left side of picture) is seen merging into the main tumor (case D 2923).

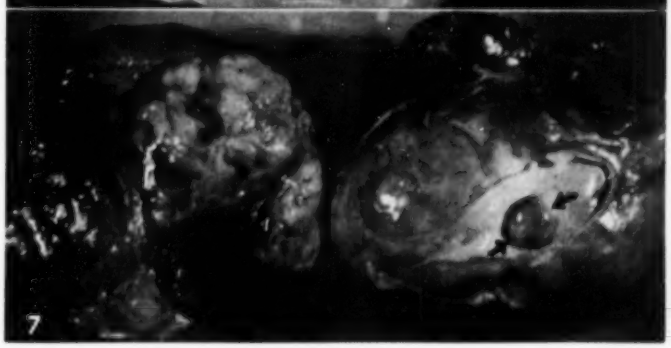


Fig. 7—Adrenal cortical carcinoma in an old cow. The tumor and remnants of an adrenal have been divided into two longitudinal sections with an incised surface of each at the left side of the picture. The remnant of adrenal is at the upper border of each section. Pedunculated tumor mass (arrows) is penetrating through the wall of the aorta (this is a border of the tumor which was adhered to the aorta, case D 2688).

thin and atrophic. On section of the tumor, it was divided into irregular yellow and orange lobules of tissue. Some of the lobules were firm, others were cystic, and most contained finely granular deposits of calcium. Areas of hemorrhage were found throughout the tumor. No metastases were found.

Squamous Cell Carcinomas of the Skin.—Since squamous cell carcinomas of the skin, with the exception of those of the orbital region, are infrequently seen in cattle, we have purposely used illustrations from 2 animals to acquaint veterinarians with their appearance. They should be differentiated from chronic ulcers and granulomas before any type of treatment is instituted. Fincher [7] recently reported the clinical observations made over a 16-month period on a squamous cell carcinoma of the skin of the maxillary region with lung and kidney metastases in a 6-year-old Holstein cow.

Unfortunately we were not able to collect smaller squamous cell tumors of the skin which had not yet developed invasive characteristics or metastases. Such would probably be amendable to treatment; however, it would not seem economically feasible to treat animals similar to the 3 found unless they represented valuable breeding stock. The necropsy reports on two tumors (fig. 8–11) indicated their large size (20 and 17 cm. in their greatest surface diameter). The third, found in an old Hereford cow, was similar in appearance and replaced an area 16 by 6 cm. It was near the right lateral border of the vulva with the tumor extending to the labium but not onto the vulvar mucous membrane. There was localized invasion of subcutaneous tissues and muscle but no metastases.

The metastatic squamous cell carcinoma listed

Fig. 8 and 9—Squamous cell carcinoma of the skin of the gluteal region with ischiatic lymph node metastases in a 7-year-old Hereford cow showing the surface (fig. 8) and an incised segment (fig. 9). Two incised sections of the tumefied ischiatic lymph node are seen (lower border, fig. 9) and arrows indicate an oval, demarcated area almost completely replaced by the tumor (case D 3151).

(table 1) as having origin in a primary site other than the eye probably arose in the skin of the lower jaw of a 5-year-old Hereford cow. Unfortunately the jaw lesion was discarded before a detailed examination and a collection of tissues from it could be made. Metastatic lesions in the posterior cervical and prescapular lymph nodes were typical of a squamous cell carcinoma.

Illustrated Cases

Figures 8 and 9.—Squamous cell carcinoma of the skin with ischiatic lymph node metastases in a 7-year-old Hereford cow is shown (case D 3151).

Necropsy.—A raised, ulcerating, firm, cauliflower growth replaced the skin of the gluteal region on one side in an area (20 x 17 cm.). Several horny projections (up to 2 cm. long) were arising from within the growth and the adjacent skin several centimeters from the border of the tumor. A linear scar, suggesting a remnant of a brand, was also seen merging into one border of the growth. The tumor was approximately 2 cm. thick, grey to yellow, and, on cross section, rather papillary in its pattern of growth. Neoplastic masses were infiltrating some of the underlying muscular tissues as linear projections which separated muscle fibers. The corresponding ischiatic lymph node was greatly enlarged (7 x 5 x5 cm.) and almost completely replaced by yellowish, often granular or fibrous tumor masses. The tumor was also seen infiltrating muscle adjacent to the lymph node in some of the area where it had penetrated the capsule of the node. The sublumbar lymph nodes were enlarged due to hyperplasia of the parenchyma of the nodes but did not contain recognizable metastases.

Figures 10 and 11.—A squamous cell carcinoma of the skin with ischiatic lymph node and lung metastases in a 3-year-old Holstein-Friesian cow is shown (case D 2267).

Necropsy.—A raised, cauliflower ulcerating growth (17 x 16 x 3 cm.) with a mushy, necrotic surface was present in the skin of the right hip. (Many maggots were present on its surface.) On cross section of the tumefied skin, a yellow, granular, papillary mass had replaced the normal epithelial surface and was invading the adjacent subcutaneous tissue and muscle. A firm, white mass (2 cm., dia.) was found in the incised right ischiatic lymph node and a similar mass (5 mm., dia.) was present in one of the diaphragmatic lobes of the lung.

Squamous Cell Carcinoma of Vulva.—Our single example of a vulvar carcinoma was an ulcerating, necrotic, and maggot-infested growth covering an area (20 x 13 cm.) in the dorsal and two lateral mucosal surfaces of the vulva of an 8-year-old Hereford cow. The involved area extended approximately to the vulvovaginal junction and was sharply demarcated from the vagina and

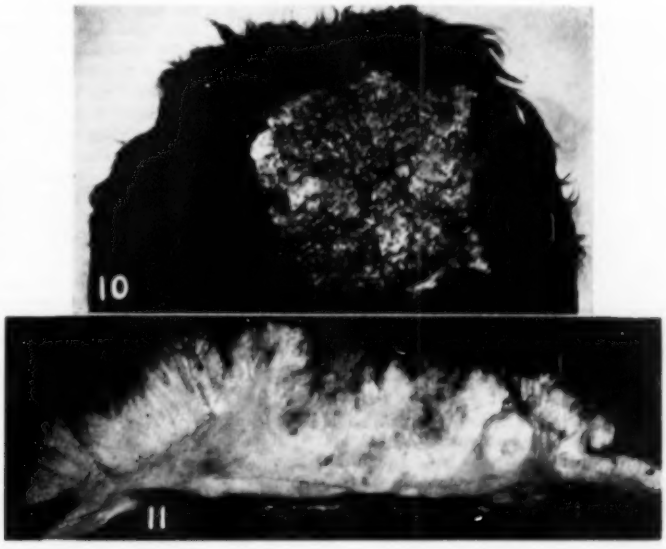


Fig. 10 and 11 — Squamous cell carcinoma of the skin of the hip in a 3-year-old Holstein-Friesian cow showing the surface of the primary tumor (fig. 10) and an incised segment with adjacent skin (fig. 11, case D 2267).

adjacent vulva by several firm, nodular, greyish growths (each about 2 cm., dia.). Posteriorly, the tumor extended onto the skin of the perineum as small nodular masses. The rectal, sacral, and ischiatic lymph nodes were enlarged due to lymphoid hyperplasia but contained no detectable tumor metastases.

Multiple Papillomas of Oral Cavity.—The lesions in this 2-year-old Hereford steer might represent on oral form of infectious papillomatosis. However, there were no associated cutaneous lesions. Raised, greyish, cauliflower, or papillary growths (11 x 6 cm. and 2 x 2 cm.) were present on the ventral and on the lateral borders of the body of the tongue and one (7 x 4 cm.) on and around the left lower incisors.

BOVINE NONEPITHELIAL TUMORS

Lymphoid Tumors.—Since much has been written and many observations made on lymphoid tumors in cattle, including an excellent chapter by Feldman,[12] we have not illustrated the many types of lesions which make these tumors spectacular and economically important.

We have adopted the designation "lymphoma" for the benign tumor and "lymphosarcoma" (malignant lymphoma) for its malignant counterpart. Sub-group terms, such as "follicular lymphoma," "lymphoblastoma," lymphocytoma," and "reticulum cell sarcomas," have value in describing or indicating the prognosis of individual cases of bovine lymphoid tumors. However, no distinct follicular lymphomas or reticulum cell sarcomas were found in our survey group; the variations in histological appearance could better be appraised in the future by the study of a larger group.

The 20 lymphoid tumors were all diagnosed as lymphosarcomas. Some of the lesions in each affected animal were malignant in their gross and microscopic appearance and lesions were found in at least two different anatomic sites in each animal. Smaller lymphoid tumors which probably would include those whose appearance might still suggest a benign stage of growth (lymphoma) were not seen but could easily have been overlooked in routine ment inspection or confused with regional lymphadenitis. Actually, there is no reason to expect more than an infrequent occurrence of benign lymphoid tumors if it can be accepted that most lymphoid tumors enlarge rapidly and produce a fatal termination in a few weeks after they are recognized clinically. Feldman[12] reported two of 61 cases of bovine lymphoid tumors to be benign or in the lymphoma class, while 59 were malignant.

A reasonably careful clinical examination should have established a diagnosis of possible lymphoid tumor prior to slaughter in all of the 20 affected animals in our survey. In 2 animals, lesions were limited to massive tumefaction of the sublumbar, renal, and internal iliac lymph nodes with extension into adjacent sublumbar tissues. Such involvement should not be difficult to detect in the living animal; however, similar lesions were observed in at least 12 of the other animals afflicted with other additional lymphoid growths which, as noted later, would be even more simple to diagnose clinically. In 2 of these animals, the sublumbar growths had infiltrated into the vertebral canal in the region of the last lumbar and the first sacral vertebrae resulting in a noticeable difficulty in the coordination of the hind legs. Multiple uterine lesions were also observed in 6 animals with sublumbar lesions. The uterine lymphosarcomas, in the living animal, could easily be confused with smooth muscle tumors, uterine carcinomas, or various granulomas. This extensive regional distribution of some of the lymphosarcomas in lymph nodes or visceral organs indicated that there may have been regional metastases as well as obvious invasive spread of the tumors. Regional metastases could occur. This would not conflict with accepted theories of origin of one or more of the tumors in the involved area from a leukemia with metastases or from growths having multicentric origins.

Most veterinarians are familiar with the massive solitary or multiple bovine lymphosarcomas of the cervical region which, as individual or confluent growths, often attain a diameter of 20 to 50 cm. Ten of our 20 lymphosarcomas, including one found in an 8-month-old Holstein-Friesian calf, were from animals with lymphoid tumors located in variable patterns from the pharyngeal region to the thoracic inlet. An effort was made to determine whether any of these growths could be identified as thymic tumors. A 1-year-old Aberdeen Angus heifer had groups of cells resembling the epithelial cells of Hassall's corpuscles scattered among neoplastic lymphoid cells in a reticular stroma in one of several growths. In still identifiable but tumefied lymph nodes in this animal, there were only neoplastic lymphoid cells. One explanation advanced for the included thymic structures was that they were atrophic remnants of thymus which had been engulfed by an expanding tumor. In our files, we have two examples each of thymic epithelial tumors of moderate size in cattle, sheep, and swine but none of these was represented by multiple or metastatic growths. It is true that lymphoid tumors could arise in the lymphoid elements of thymic tissue but it is confusing to call them thymomas.

Two of the lymphosarcomas were from animals with lesions in the orbital region; one was in the membrana nictitans, the other in the conjunctiva of the eyelids. The supramammary lymph nodes were markedly tumefied in 1 affected animal; one tumor mass was 30 cm. in its greatest diameter. Ten animals had heart lesions and 6 had massive growths in the wall of the forestomachs. One of the latter was affected with chronic bloat, observed on antemortem inspection.

No animal had metastases in both the lung and liver but such might have occurred later if the animals had not been slaughtered. Three had multiple metastatic lesions in the lung but none

in the liver or kidney while 6 had multiple growths in the liver and no recognizable lung metastases. No studies were made of the blood to determine if any type of leukemia existed. Feldman's observation,[15] that the spleen was not demonstrably involved in many of his cases (14 tumefied spleens in 40 animals), was supported by our findings of definite lesions in only five spleens in the 20 animals.

The 20 cattle were of all age groups; 3 were 6 years old, 2 each were 1, 2, and 7 years old, 1 each were 3, 4, 8, 9, and 12 years old, 5 were listed as old animals, and 1 was an 8-month-old calf. The group consisted of 1 bull, 2 steers, and 14 females. The breed recorded for 12 animals was: Holstein-Friesian, 5; Hereford, 5; and Aberdeen Angus, 2.

Although no bovine myeloid tumors (chloromas) were collected in the survey, they should be mentioned because they have been rather frequently submitted to this laboratory and might be confused grossly with lymphoid tumors. Attention is directed to them either because of a widespread distribution in lymph nodes, the greenish color of most, or their unusual appearance when present in skeletal muscle, abdominal viscera or, more rarely, in bone. They are usually quite distinctive microscopically and are not difficult to differentiate from several types of focal myositis of skeletal muscle, which may be green, characterized by massive infiltrations of eosinophils, and of much more frequent occurrence. In the myeloid tumors, neoplastic myeloblastic cells, and cells with cytoplasmic granules, which are indistinguishable from mature granular leukocytes, are frequently seen in the same lesion. We have been puzzled by a tumor observed once in skeletal muscle and once in the spleen in which the neoplastic cells of the lesions were morphologically similar to mast cells in various stages of differentiation. Whether these two tumors are true mast cell sarcomas or myeloid tumors is debatable.

Schwann's Cell Tumors.—Schwann's cell tumors in cattle would seem to be increasing in frequency in the United States. Even though the 43 cases (table 1) made the Schwann's cell tumor the most frequently diagnosed bovine tumor, it is a certainty that other schwannomas were overlooked. Many of the anatomic sites in which the growths are found, and particularly the peripheral innervations of the musculature, can not be examined in routine meat inspection.

No special search was made for lesions of this disease. We did check, when possible, for multicentric manifestations of the tumor at necropsy when one or more suspected Schwann's cell growths were found. Multiple neoplastic lesions were confirmed by microscopic examination in 30 of the 43 affected animals. Circumstances did not permit the conducting of detailed necropsies in the 13 animals with solitary lesions and in 15 of the 30 animals with multiple lesions. Findings in these 28 cases, in which no search was made for additional Schwann's cell tumors, would be representative of those made by meat inspectors in routine examination of viscera and exposed portion of carcasses.

Indications of metastasis were not apparent in any of the 43 cases and most of the lesions appeared quite benign. In an earlier paper[20] from this laboratory, it was suggested that Schwann's cell tumors frequently had multicentric origins in affected animals.

The cattle in the present group were older than those discussed in the 1953 paper on Schwann's cell tumors. Of the 43, 27 were listed only as old animals, 1 each as 3, 9, 10, and 12 years old, 3 as 6 years old, 3 as 7 years old, 4 as 8 years old and 2 had no recorded age. All were cows, except for one 3-year-old steer and 2 old bulls. Of the 27 affected animals, 21 were Herefords, 1 a Holstein-Friesian, 1 an Aberdeen Angus, 1 of mixed breed, and for 19 the breed was not reported.

The clinical appearance of cattle with Schwann's cell tumors is beginning to be recognized in the United States. Goodman *et al.*[17] in New York recently made clinical observations on 2 affected cows; the multiple tumors encountered in the subsequent necropsies represented types of growths consistent with those previously described.[20]

Since the pathology of bovine Schwann's cell tumors was discussed and illustrated in 1953,[20] only a few additional observations on gross appearance and sites of origin will be made: One case (D 2231) in the present two-year survey had been diagnosed in January, 1953, and was included with 24 other cases in the 1953 report. There was no other overlapping of the two collections of Schwann's cell tumors.

In one case in the present study, outgrowths

Fig. 12—Tumefactions of a seventh cervical spinal nerve by a Schwann's cell tumor in an old Hereford cow. The tumor involved the dorsal and ventral root fibers (arrows) near the spinal ganglion but did not extend to the spinal cord (case D 2816).

Fig. 13—Cut surfaces of two encapsulated Schwann's cell tumors attached to a nerve of the brachial plexus in a 10-year-old black cow of mixed breed. Note the cystic spaces in the larger tumor in the upper portion of the photograph (case D 2863).

from tumefied posterior cervical and first thoracic ganglia were traced to growths involving the spinal nerves. These growths extended to enlarge some of the spinal ganglia (up to 2 times) and even the dorsal and ventral nerve root fibers between these spinal ganglia and the spinal cord (fig. 12). The tumor did not directly involve the spinal cord or the segments of the dorsal and ventral root fibers immediately adjacent to the cord.

The large cystic spaces described as a frequent component of Antoni type-B tissue in human Schwann's cell tumors were not observed in the 1953 study but, in the present two-year survey, they were seen in growths distinctly attached to nerves. This type of cyst is usually identifiable at necropsy (fig. 13). We have not seen the cysts in the heart and this is logical when it is realized that the cysts represent an aging phenomenon in an original Antoni type-A tissue growth which is seldom present in the cardiac tumors.

Among the 43 cases in our present survey, we observed 30 in which there were single or multiple Schwann's cell tumors of the heart; 23 were multicentric growths. A review of the 16 cases with cardiac involvement reported in 1953[28] and the data on the present cases have made us more certain that the growths originate in the sheaths of branches of cardiac nerves. As mentioned in 1953, the lesions are often poorly encapsulated and may be found several centimeters from a cardiac nerve or its main branches. However, so many of the tumors are found in the epicardium or myocardium in or near the left or right longitudinal grooves (fig. 14) that one would suspect they had some relation to the larger nerves or blood vessels. Frequently, only one groove or a portion of a groove is associated with a group of multiple growths.

Branches of the cardiac nerve could be the point of origin for a myocardial growth but we have not found a plexiform tumefaction of such a nerve extending from the tumor to involve large branches of the cardiac nerves. In a few instances, small superficial epicardial tumors were attached to or were actually tumefied portions of small epicardial branches of the cardiac nerves. These tumors seemed to be similar to those observed by

Fig. 14—A lobulated, encapsulated Schwann's cell tumor arising in the epicardium and myocardium of the right ventricle in a 6-year-old cow (case D 2700).

Goodman *et al.*,[17] who reported many instances of a definite attachment of Schwann's cell tumors to epicardial branches of cardiac nerves in two cases. However, the majority of our Schwann's cell tumors of the heart were not superficial but embedded deep in the myocardium. Some were completely surrounded by muscle and were not visible until the heart was incised. Others appear to have originated in the myocardium and to have invaded the muscle until they were visible beneath unincised epicardium. The point of origin in a nerve for these two types of growth has not been established, although small nerves have been found in them. There is no doubt about the extracardial involvement of cardiac nerves in the cases with that type of involvement which we have observed; these growths are consistently attached to nerves and are predominantly composed of Antoni type-A tissue. When located within the myocardium, the tumors are preponderantly of a cellular, haphazardly arranged schwannian tissue;[20] this is unfortunate because it is the Antoni type-A

Fig. 15—A pedunculated Schwann's cell tumor attached by a stalk to the endocardium and myocardium of the left ventricle in an old Hereford cow. A second Schwann's cell tumor was found in the myocardium of this same ventricle (case D 2874).

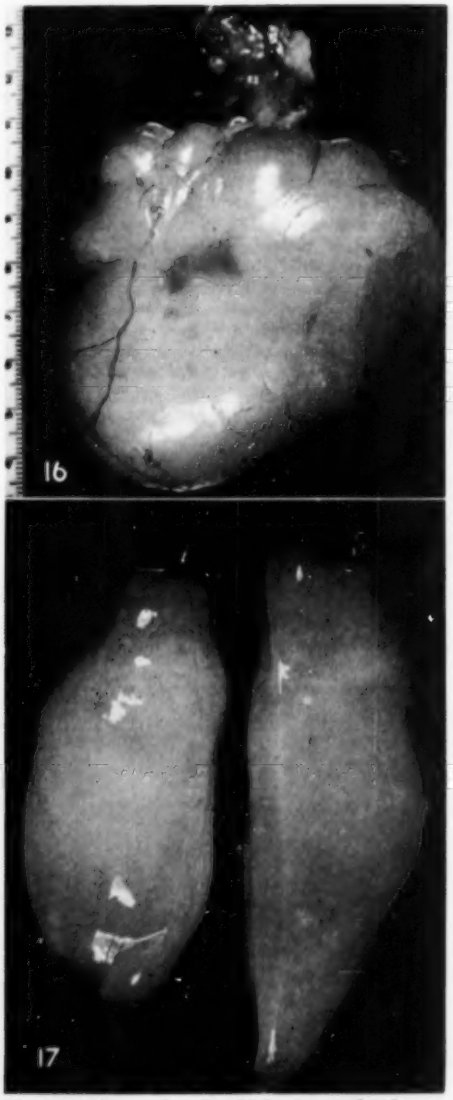


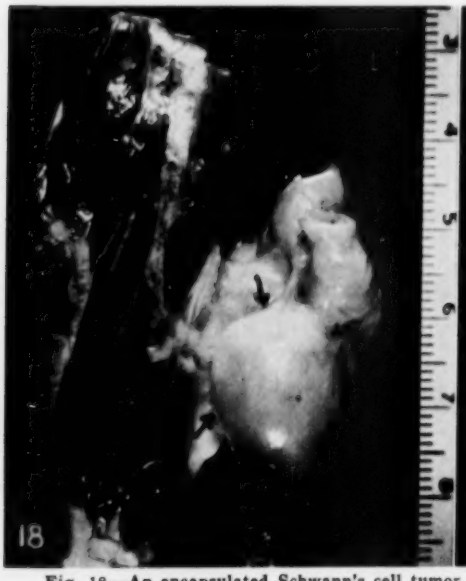
Fig. 16 and 17—A soft, thinly encapsulated, mucinoid Schwann's cell tumor which was attached to a branch of a tumefied spinal accessory nerve in the musculature covering the posterior lateral surface of the scapula in an old Aberdeen Angus cow. Figure 16 shows the exterior of the tumor and figure 17 represents two incised surfaces of the growth. Multiple Schwann's cell tumors of the heart, brachial plexuses, and cervical, thoracic, and abdominal ganglia and plexuses were also found in this animal (case D 2734).

growths in which one can most clearly demonstrate a close attachment of tumor to nerve sheaths in other anatomic locations in cattle. We have seen occasional examples of growth of Antoni type-A tissue in some of the larger epicardial schwannomas. A point of origin in a nerve can be demonstrated conclusively in a few of these epicardial growths but not in the myocardial tumors or in endocardial growths.

In this survey, five polyp-like growths arising in the endocardium (fig. 15) have been established as Schwann's cell tumors. At first, we believed that most of these were fibromas which are similar in appearance and occur in this same anatomic site. However, we found a microscopic pattern in parts of each tumor which was typical of neoplastic Schwann's cell tumors. In 4 of the 5 animals, one or more Schwann's cell tumors were also found in the myocardium of affected hearts and 2 of them had additional Schwann's cell tumors in other anatomic locations.

The Schwann's cell tumors attached to nerves of skeletal muscle may vary greatly in their consistency and appearance. However, excluding the plexiform group, most are composed of Antoni type-A tissue. A soft, mucinoid, loosely textured, Schwann's cell tumor (an unusual tumor) is illustrated (fig. 16, 17) to show that extreme variations in appearance may occur in these tumors of peripheral nerves. A more typical tumor of the firm, nodular, neurilemmoma type of growth (fig. 18) is apparently also the most frequently encountered type of Schwann's cell tumor in man.

In the nine cases of Schwann's cell tumors reported by Jackson[10] in 1936, two with lesions supposedly limited to the tongue were described. Careful examination was made, therefore, of the tongue in all necropsies in the present study and particularly in all animals with lesions suspected to be Schwann's cell tumors. Two animals (both in this survey) with growths attached to the muscle fibers of the tongue have been found. One soft, greyish, poorly encapsulated, mucinoid tumor (30 x 25 x 25 mm.), embedded deep in the musculature in the region where the dorsum linguae forms its posterior prominence, was found in a 10-year-old, black, mixed breed female. The other was in an old Hereford cow. Numerous, greyish, fibrous nodules (5–15 mm. their greatest diameter) were found in the musculature at the base of the tongue; some of these were visibly attached to small nerves. Many additional growths were found in various anatomic sites in this animal; they included several tumors attached to nerves in the fascia of the omohyoideus and sternocephalicus muscles (fig. 18) and one extensive growth involving one adrenal ganglion and much of the medullary portion of the same adrenal. Perhaps, the most unusual neoplastic lesion in this animal was represented by the third unilateral acoustic Schwann's cell tumor diagnosed at this laboratory. In the two acoustic tumors mentioned in 1953,



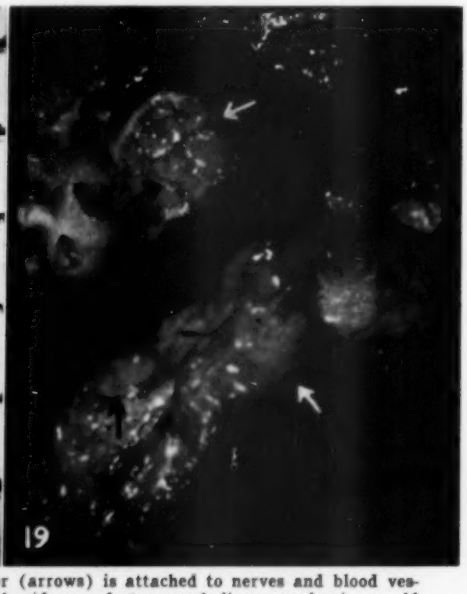


Fig. 18—An encapsulated Schwann's cell tumor (arrows) is attached to nerves and blood vessels in the fascia near the insertion of the omohyoideus and sternocephalicus muscles in an old Hereford cow.

Fig. 19—Schwann's cell tumors (arrows) are attached to nerves in the parenchyma of the liver near the walls of the portal vein in the same animal (case D 2817).

Fig. 20—An extensive plexiform tumefaction by a Schwann's cell tumor of three nerves of the brachial plexus in a 7-year-old Hereford cow (case D 2577).

clinical signs of a central nervous system disturbance had been observed, but not in the present case. The acoustic tumor was a fibrous, lobulated growth (6 x 3 x 3 mm.) which was distinctly attached to the border of the left acoustic nerve about 15 mm. peripherally from the point where its roots merge as a single nerve. On microscopic examination, the lesion was found to be a neurilemmoma type of Schwann's cell tumor with Antoni type-A tissue and distinct Verocay bodies.

Plexiform Schwann's cell tumors are most spectacular at necropsy when involving larger nerves (fig. 20) but one has no difficulty in immediately sensing the abnormality in small nerves. By careful palpation, one may attempt to find the small nodular and frequently associated growths of Antoni type-A tissue (neurilemmoma) to help confirm a gross diagnosis of an early plexiform growth. Nerves of the brachial plexus can be easily palpated when exposed at necropsy. The smaller of the two incised nodular growths attached to a tumefied nerve (fig. 13) is an example of this type of involvement.

Enlarged nerves can usually be seen extending from the borders of ganglia which are tumefied with schwannian growths. Some tumefied ganglia (fig. 21) are more identifiable than others. A tumefied coeliac plexus may become greatly enlarged and continuous with the hepatic plexus. In one case (fig. 19), a tumor of these plexuses followed the ramification of numerous nerves into the parenchyma of the liver.

Adrenal Medullary Tumors.—The adrenal medul-

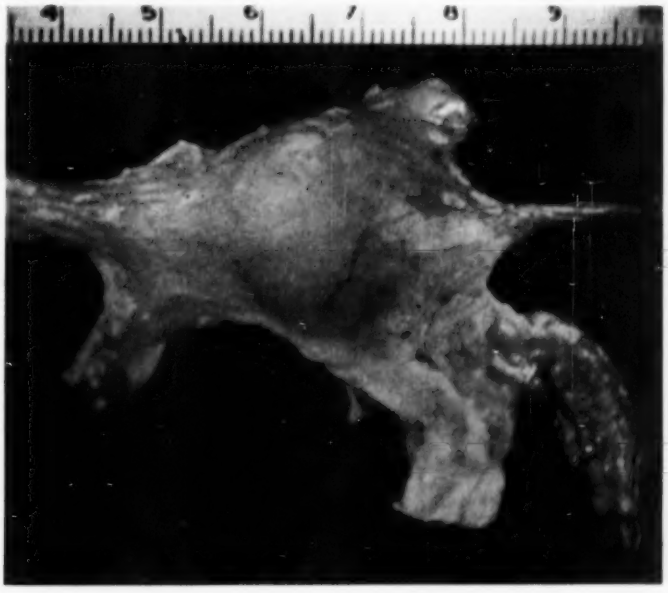


Fig. 21—A schwannoma of the first thoracic ganglion with plexiform tumefactions of the nerves communicating with the ganglion in an old Hereford cow (case D 2731A).

lary tumors were represented by two different types of tumors. Among our five tumors, three were considered to be neuroblastomas and two were pheochromocytomas (chromaffin tumors).

Of the three neuroblastomas, two were found in young animals, 1 and 4 years old. The third was 10 years old and all were females. In the 2 younger animals, the tumors were large; one from each was 30 cm. in diameter and a second bilateral tumor in the 4-year-old was 5 cm. in diameter. In the 10-year-old, two small growths (13 and 15 mm., dia.) were found in the medullary zone of one adrenal and represented an incidental finding in a detailed necropsy to study the distribution and extent of Schwann's cell tumors of the brachial plexuses, tongue, heart, mediastinum, and abdominal ganglia, nerves, and plexuses. All of the neuroblastomas, microscopically, were composed of neuroblastic cells and formed rosettes in some portions of the growth. In our survey, we did not see any bovine ganglioneuromas of the adrenal which, histologically, are recognized by more mature appearing neoplastic nerve cells and fibers and usually include appreciable numbers of recognizable ganglion cells. Neither did we observe mixed ganglioneuromas and neuroblastomas, which are believed to represent varying degrees of differentiation within a tumor of neuroblastic cells. These bovine mixed tumors and ganglioneuromas have been diagnosed in other abattoir specimens submitted to our laboratory.

The two pheochromocytomas were diagnosed histologically by the resemblance of their component cells, both in morphology and arrangement to adrenal medullary tissue. Some of the tumor cells had brown-staining granular cytoplasm when fresh blocks of tissue were fixed in a special chromate solution. However, these cells were not numerous in either of the tumors and most of the tumor cells in each had only a few scattered granules associated with light chromaffin staining of the cytoplasm. Similar bovine tumors, in our files, which had been fixed in chromate salts generally stained poorly for chromaffin. Perhaps, many of the tumors were not differentiated sufficiently to form large numbers of granules. Both were in old cows and 1 animal had a bilateral involvement. One of the tumors, in the latter cow, was 10 cm. in diameter with a rounded, thinly encapsulated projection (2 cm., dia.) protruding through the wall and into the lumen of the posterior aorta. This tumor was adherent to the kidney and liver. The tumor in the opposite adrenal was only 1 cm. in its greatest diameter, was surrounded by medullary tissue, and was identical in microscopic appearance to the other tumor. The pheochromocytoma in the second cow was 11 cm. in its greatest diameter.

Smooth Muscle Tumors.—The eight smooth muscle tumors were solitary, benign leiomyomas. Four were present in the wall of the uterus, two in the myocardium, one in the anterior mediastinum, and one in the parenchyma of the lung. As a group, the leiomyomas were rather massive and encapsulated. The unusual growth in the lung of a 1-year-old steer was 9 cm. in diameter. Both heart tumors were 5 cm. and the mediastinal one was 2 cm. in diameter. The leiomyoma shown in figure 22 was not our largest uterine growth as another in an old cow measured 28 by 18 by 7 cm. One 11 by 10 by 6 cm. was found in a 10-year-old cow in a gravid horn which contained a nearly full-term calf. Whether the tumor had been detected clinically is not known since no history was available. The fourth uterine leiomyoma was 13 cm. in diameter. Ovarian cysts were found in 3 of the animals with uterine leiomyomas.

The breed of 5 had been recorded; all were Herefords; all were females except for the one steer; and 3 were listed as old, 2 as 10 years old, and 1 each as 1, 2, and 6 years old.

Illustrated Case

Figure 22.—Leiomyoma of the uterus of an old Hereford cow is shown (case D 2675).

Necropsy.—A solid, oval, thinly encapsulated mass (14 x 14 x 12 cm.) was present in the wall of the body of the uterus, with one border within 1 cm. of the cervix. Neither the mucosa nor the serosa was penetrated by the tumor. On section, the tumor was firm and, in some areas, fibrous. It was grey and had yellow necrotic areas scattered in it. One ovary had a large cyst in its parenchyma.

Angioid Tumors.—Five angiomas and 1 angiosarcoma were found. We had not only the difficult problem of differentiating benign (angioma, hemangioma, hemangio-endothelioma) from malignant (angiosarcoma, malignant hemangioma, and malignant hemangio-endothelioma) but also had to attempt to identify vascular malformations and tumors of these malformations (we prefer to designate a vascular malformation as a hamartia and its benign neoplastic form as a hamartoma). The identification of angioid tumors as hamartomas is almost impossible to make except in the young or where there is a still recognizable congenital malformation. They were found in younger animals (3 were 2 years old, 1 was 3 years old, and 2 were 5 years old); 4 were steers and 2 were cows; 5 were Herefords; and all appeared to be in good condition at slaughter so the tumors were believed to represent incidental findings.

Angioid tumors can be multicentric in their origin and it is difficult to determine whether multiple and anaplastic growths such as were seen in heifer D 2871 (fig. 23) were actually multiple tumors or a single primary tumor with metastases. Although the tumors appeared locally invasive in this case and there may have been a metastasis of some of them by extension in the regional lymphatics, lack of liver or lung metastases indi-

cated that the tumors probably arose independently.

The other five were regarded as benign angiomas and were represented by single lesions; two were in the spleen (fig. 25, 26), two in the myocardium of the ventricles just beneath the endocardium (fig. 24) and one in the anterior mediastinum. The angiomas of the heart had a tendency to form cavernous spaces; one was found in the left ventricle, the other in the right ventricle. The splenic angiomas contained numerous cysts, many filled with blood from intracystic hemorrhages. There are angioid tumors of the liver, lung, kidney, and skin in our collection but none was encountered in those sites in this survey.

Illustrated Cases

Figure 23.—Angiosarcoma of the parietal peritoneum, mesentery, omentum, and internal iliac, bronchial and mediastinal lymph nodes is shown in a 2-year-old Hereford heifer (case D 2871).

Necropsy.—Multiple red and firm or cystic tumors were found on the parietal peritoneum, omentum, and mesenteries. They varied in size from 1 to 30 cm. in their greatest diameter and were not present on the abdominal organs. They were quite irregularly shaped, and some-

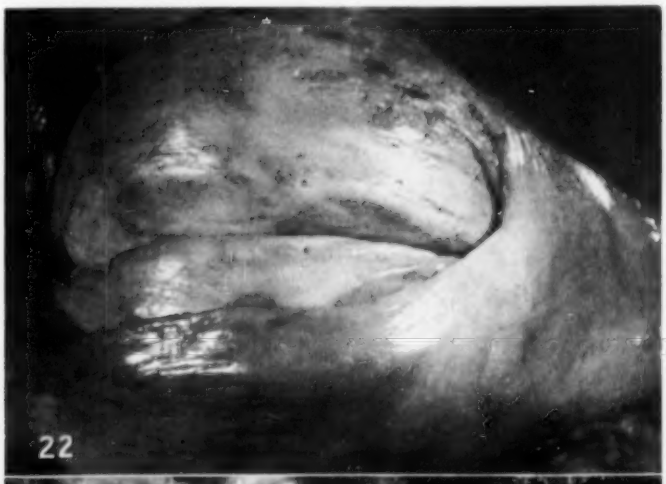


Fig. 22 — Leiomyoma of the uterus in an old Hereford cow. An incision has been made through the originally intact serosa into the main tumor mass (case D 2675).

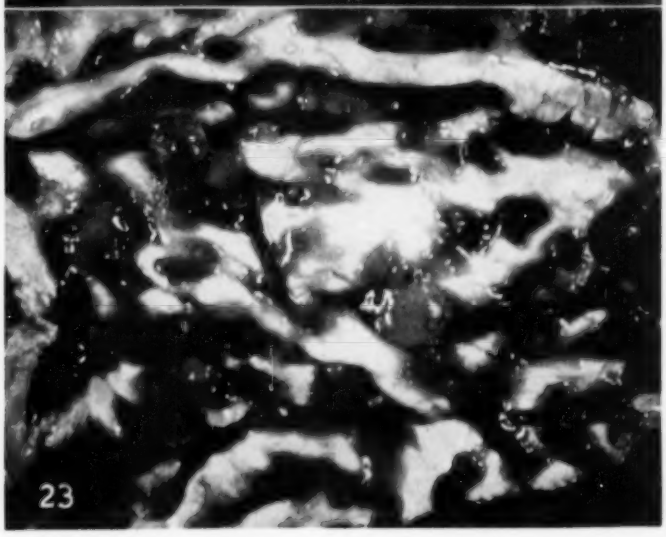


Fig. 23 — Angiosarcoma of the omentum in a 2-year-old Hereford female. Several cysts can be seen (case D 2871).

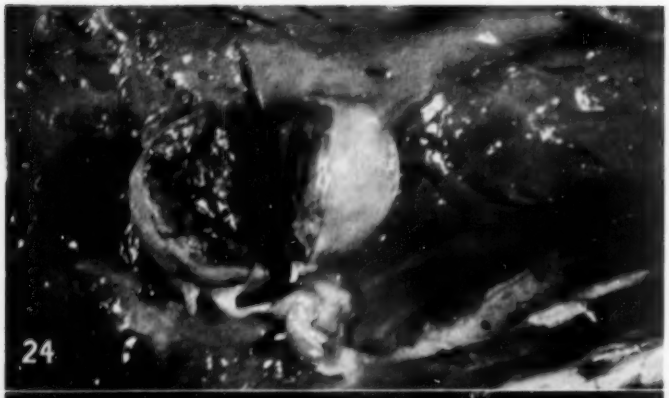


Fig. 24—Angioma of the endocardium and myocardium in a 3-year-old Hereford steer. The tumor has been incised (case D 2804).

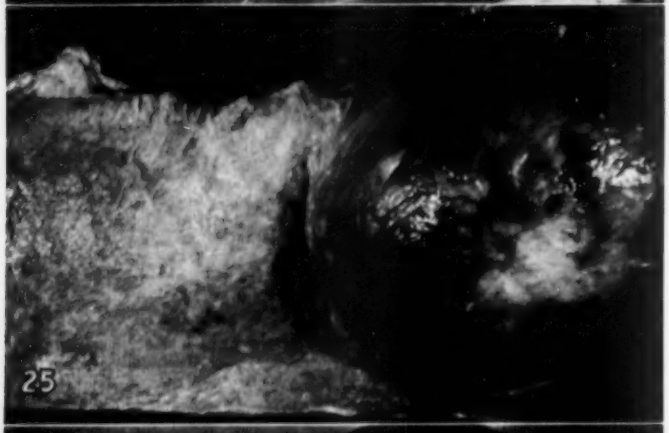


Fig. 25—Angioma of the spleen in a 2-year-old Hereford steer (case D 2738).

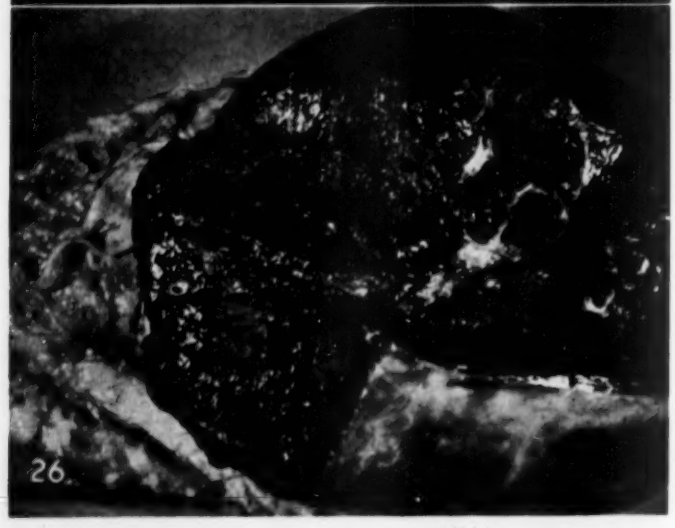


Fig. 26—Angioma of the spleen in a 5-year-old Hereford steer. About three fourths of the surface of the tumor has been removed to show a partial cross section of the growth (case D 2787).

times portions of a single tumor were connected only by a narrow strand of neoplastic tissue. Firm, reddish tissue, interspersed with small blood-filled spaces, had replaced portions of enlarged internal iliac, bronchial, and mesenteric lymph nodes. The uterus of this animal contained a normal-appearing fetus estimated to be 6 months of age.

Figure 24.—Angioma of the heart of a 3-year-old Hereford steer is shown (case D 2804).

Necropsy.—An oval, firm, encapsulated, glistening mass (30 x 20 x 14 mm.) protruded from the endocardium of the right ventricle. The base of the tumor merged into the superficial layers of myocardium beneath the endocardium with much of the point of attachment being confined to a prominent ridge of one of the trabeculae carneae. On section, there were small blood-filled spaces arranged between firm, reddish and softer, pale yellow tissue. A few, small areas of calcification were present in the growth.

Figure 25.—Angioma of the spleen of a 2-year-old Hereford steer is shown (case D 2738).

Necropsy.—A single, oval, thinly encapsulated mass (14 x 12 x 7 cm.) protruded from the parietal surface of the spleen with one border extending to the hilus and another to within 2 cm. of the dorsal extremity. The surface of the tumor was roughened, and small membranes from former adhesions could be stripped from it. On section, the firm, reddish or soft, pale yellow tissues of the growth were divided irregularly by dense, fibrous trabeculae. Cystic spaces, usually several millimeters in diameter, containing blood or clear yellow or pinkish fluid were present in the tumor.

Figure 26.—Angioma of the spleen of a 5-year-old Hereford steer is shown (case D 2787).

Necropsy.—An oval, thinly encapsulated growth (15 x 14 x 7 cm.) arose from the visceral surface of the spleen and one border of the tumor extended to within 2 cm. of the spleen's ventral extremity. On section, the growth was a firm, reddish, indistinctly lobulated mass. Several, thinly walled cystic spaces (up to 1 cm. dia.) filled with blood or red, watery fluid were present within the tumor.

Fibrous Connective Tissue Tumors.—The differentiation of a benign fibroma from a fibrosarcoma is often difficult or impossible. There is a tendency to regard many fibrous connective tissue tumors as fibrosarcomas and this is partially justified when one encounters the not too infrequent case with metastases. The clinical behavior of fibrous tumors in specific sites, as indicated in the following paragraphs, is often more helpful than microscopic findings in making a diagnosis of a benign or malignant tumor.

Of our seven tumors, three were diagnosed as fibrosarcoma when their histologically anaplastic type of growth showed pronounced gross and microscopic invasive tendencies. One was a primary fibrosarcoma in the subcutis and muscle of the cheek (fig. 29) with parotid lymph node and

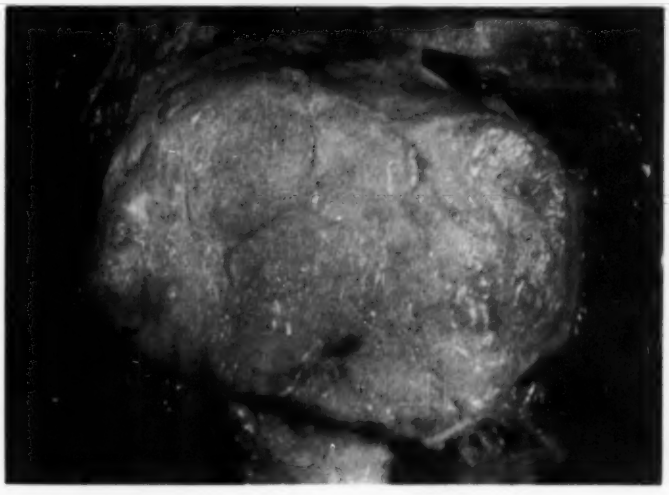
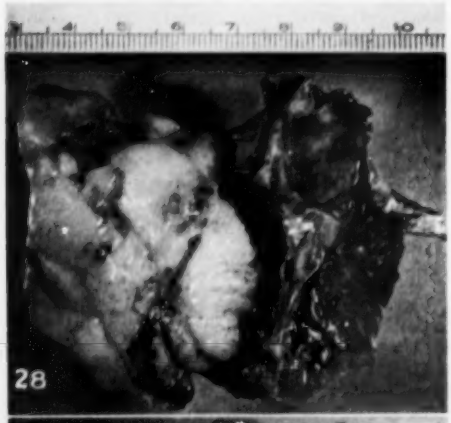


Fig. 27—Cut surface of a fibroma of the abdominal wall in an old Hereford cow (case D 2187).

lung metastases; the second was a subcutaneous mass around one button remnant of an undeveloped horn in a 6-year-old Aberdeen Angus cow, and the third was in the frontal sinus (fig. 28).



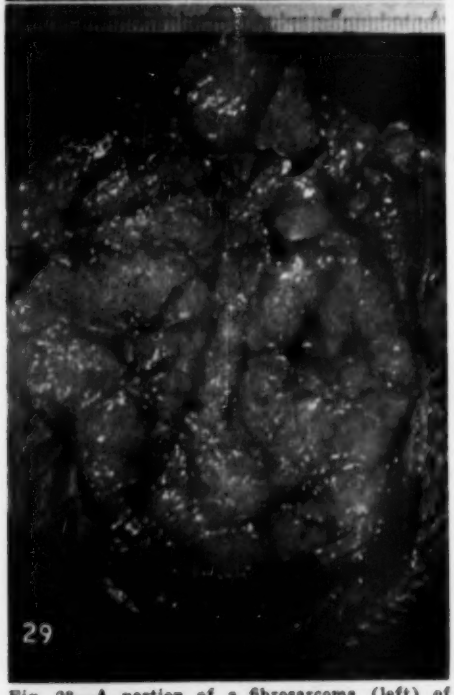


Fig. 28—A portion of a fibrosarcoma (left) of the frontal sinus in a 5-year-old Hereford cow. The outer segment of the tumor was attached to the sinus mucosa by adhesions only (case D 2367).

Fig. 29—Cut surface of a fibrosarcoma of the cheek with parotid lymph node and lung metastases in a 4-month-old Hereford heifer (case D 2182).

A large and multiple fibroma of the penis of a 2-year-old steer was diagnosed as benign on the basis of our study of several tumors and the work summarized and expanded on by McEntee,[28] which indicated that this type of tumor is not known to metastasize, but may regress and, perhaps, is initiated by the skin papilloma virus. Our other three fibromas were found in sites where we had previously observed benign fibrous tumors, the tongue, the abdominal wall (fig. 27), and the endocardium of the right auricle as a polyp-like growth.

All of the affected animals, except the steer with the penile fibroma, were females. Two were old, 1 was a 4-month-old Hereford calf, and the others were 3, 5, 6, and 8 years old.

Illustrated Cases

Figure 27.—Fibroma of the abdominal wall in an old Hereford cow is shown (case D 2187).

Necropsy.—An irregularly lobulated, white, subcutaneous growth (6 x 5 x 3 cm.) was embedded in the fascia and obliquus abdominis externus muscle of the right abdominal region. It was not attached to the skin. On section, the tumor was firm and fibrous and had only small vascular channels within it or at its periphery.

Figure 28.—Fibrosarcoma of the frontal sinus in a 5-year-old Hereford cow is shown (case D 2367).

Necropsy.—The right frontal sinus was almost completely filled with a massive, irregularly lobulated tumor which was firmly attached to or embedded in the mucosa. Portions of this growth were fibrous and a uniform greyish white. The minor compartments of the frontal sinus around the right eye also contained confluent, lobulated masses of the tumor, and these were also difficult or impossible to strip free from the underlying mucous membrane.

Figure 29.—Fibrosarcoma of the cheek with lung and lymph node metastases in a 4-month-old Hereford heifer is shown (case D 2182).

Necropsy.—A greyish white, irregularly lobulated mass (11 x 7 x 4 cm.) was present in the right maxillary region extending from the angle of the lip under the skin and almost to the border of the eye and the parotid lymph nodes and glands. Some lobules of the growth were very vascular. The tumor penetrated neither the skin nor the mucosa and was embedded in the fascia and musculature. A firm nodule about 3 mm. in its greatest diameter

was found just beneath the pleural surface of one apical lobe of the lung. A grey, fibrous mass was seen in the parenchyma of the right parotid lymph node. Tiny, soft, greyish lesions in the cortex of a greatly enlarged right prescapular lymph node represented lymphoid hyperplasia.

Mesotheliomas.—There is a hesistancy in diagnosing a primary mesothelioma of either the pleural, pericardial, or peritoneal cavity because of the similarity of lesions of some inflammatory processes and metastatic or primary tumors of serous membranes. In some peritoneal metastases of ovarian, uterine, bile duct, and intestinal carcinomas, individual histological sections could easily be mistaken for mesotheliomas.

In this survey, we found two mesotheliomas of the peritoneum and none of the pleura or pericardium. Several suspected mesotheliomas were discounted after complete necropsy or the study of numerous histological sections. Histologically, the anaplastic mesothelial cells may appear as a squamous, cuboidal, columnar, or stratified epithelium on the external portions of the tumors. The most consistent feature of most bovine mesothelial tumors is the subserous and usually irregularly papillary proliferation of connective tissue and collagen. Nests of neoplastic mesenchymal cells are frequently found within masses of this fibrous tissue but they always appear as trapped groups of cells and it would seem probable that connective tissue had grown over and around them.

In one of our survey animals (a 5-year-old cow of unknown breed), the lesions were found only on the peritoneum, omentum, and the serosa of the spleen. The other animal (a 6-year-old Shorthorn) had lesions in these same sites in addition to numerous growths on the serosal surface of the rumen and reticulum. As in other mesotheliomas studied at this laboratory, there was no evidence of metastasis from these serous surfaces.

Illustrated Case

Figure 30.—Mesothelioma of the peritoneal cavity of a 5-year-old cow is shown (case D 2922).

Necropsy.—Many greyish, firm, flattened, or papillary growths (usually 3–6 cm. in their greatest diameter and a few up to 20 cm.) covered, and were firmly attached to, large areas of the peritoneum. They were also found as multiple growths in the serosa of the rumen, reticulum, and spleen.

Melanomas.—Both melanomas found in the survey were large, contained an abundance of melanin pigment, and were found in the skin and subcutis. Neither had penetrated externally through the skin. One was benign, the other malignant with metastases.

The benign melanoma was present on the forearm (fig. 31). The malignant tumor was an infiltrating growth (13 x 10 x 3 cm.) in the skin and subcutaneous tissue overlying the left parotid salivary gland with a large metastatic lesion in the left parotid lymph node and many metastases in the cervical lymph nodes and in all lobes of the lung.

Both were in Hereford cows, 2 and 7 years of age.

Illustrated Case

Figure 31.—Melanoma of skin of the right forearm in a 2-year-old cow is shown (case D 3162).

Necropsy.—A raised tumor (13 x 10 x 3 cm.) which had a wrinkled, hairless, and

Fig. 30—Scattered mesothelial tumors embedded in the peritoneal surface of the diaphragm in a 5-year-old cow (case D 2922).

mottled black and grey surface was present on the medial aspect of the right forearm. On section, the growth was fibrous, deep brown to black, and of a uniform consistency. There was no evidence of invasion of the underlying musculature by this tumor.

Lipoma.—A solitary lipoma found in the survey was considered to have a possible congenital or hamartial origin. It was represented by a solid, flattened, encapsulated, subpleural growth (35 x 30 x 10 mm.) in one apical lobe of the lung of a Hereford heifer, 2 years old. On section, it was grey and had a fatty consistency. Histologically, the tumor was composed of fat cells in varying stages of differentiation in a fibrous stroma.

Meningioma.—Our single case of a meningioma was found in a 1-year-old Hereford female which appeared blind and circled to the left at antemorten inspection. The only lesions found at necropsy which might account for this action were noticeably thickened, reddened, and adherent leptomeninges over the cerebellum. Histologically, the involved meninges were represented by a tumor of plump, polyhedral, epithelial-like cells growing in clumps or in a distinct papillary arrangement in a very vascular connective tissue stroma.

Fig. 31—A benign melanoma removed from the medial aspect of the right forearm of a 2-year-old Hereford cow showing the incised bottom portion of the tumor (b) and the top of the growth (a) (case D 3162).

OVINE EPITHELIAL TUMORS

Adenocarcinomas.—As indicated in table 2, a primary site could be definitely determined in only two of the seven ovine adenocarcinomas reported in the survey. All of the adenocarcinomas were found in old ewes and only a thyroid adenocarcinoma (7 x 5 x 3 cm.) was not metastatic. The large adenocarcinoma of the kidney (18 x 6 x 5 cm.) had liver, lung, and mediastinal, hepatic, sublumbar, and internal iliac lymph node metastases.

Four of the five adenocarcinomas in which we could not establish a definite primary site had widespread peritoneal and abdominal visceral metastases. It was suspected that the primary site for these tumors was some portion of the urogenital tract or mammary gland. Unfortunately, the identification of these organs after evisceration could not be made due to the slaughtering methods employed in the Denver abattoirs.

In the other metastatic adenocarcinoma, lesions were found in the pleura, mediastinal lymph nodes, and lung and were regarded as metastases from either an intestinal or a urogenital tract carcinoma.

Liver Tumors.—In comparing the liver tumors of sheep with those of cattle, there were many similarities and some differences. Six of the eight tumors were liver cell carcinomas, one was a liver cell adenoma, and one was a bile duct carcinoma. One liver cell carcinoma had a single metastatic lesion in the kidney. The bile duct carcinoma was a large solitary tumor (8 x 6 x 5 cm.).

Five of the six liver cell carcinomas were represented by a single tumor. Their greatest diameters were 22, 17, 15, 11 and 8 cm.; the one with the kidney metastasis was 17 cm. in diameter. The sixth carcinoma had two lesions (11 and 1 cm., dia.) which were well separated from each other in the dorsal lobe of the liver. No hepatic cirrhosis was associated with any of the tumors and the growths either appeared slightly invasive or were thinly encapsulated. The tumor in 1 old male animal had large fibrous and calcified areas within it but no cirrhosis of the adjacent liver; the other liver cell tumors had a soft, fatty consistency.

Small areas of hematopoiesis were scattered throughout four of the five liver cell carcinomas, the one liver cell adenoma and, to a lesser extent, in the liver parenchyma adjacent to some of the tumors.

The variety of color changes seen in the bovine liver cell tumors were not present in the sheep tumors. A very noticeable deposit of fat in the liver cell tumors and not in the nontumefied portions of the liver gave all but one of the growths a grey to yellow color with the associated hematopoiesis producing reddish, punctate markings on this light background. One tumor had grey, fatty lobular areas alternating with light brown lobules.

Small areas of hemorrhage and necrosis were present in all the tumors but were not prominent.

There was no uniformity in the histological appearance of the sheep tumors to compare with that seen in the bovine liver cell carcinomas. A trabecular structure was observed in each growth but the embryonal, immature, and mature cell type structure of some groups of neoplastic cells would place at least one growth in each of Edmondson and Steiner's [17] four grades of liver cell carcinoma with trabecular structure. Invasiveness and cells with large intensely hyperchromatic nuclei not infrequently arranged as multinucleated giant cells helped to establish the metastatic and one other carcinoma as particularly malignant tumors.

The age of 4 of the animals afflicted with liver cell tumors, including the 1 diagnosed as an adenoma, was estimated to be between 8 months and 1 year. The other 3 were 2 old ewes and an old ram. The bile duct carcinoma was found in an old ewe.

Adrenal Cortical Tumors.—It had been expected that a high percentage of the sheep tumors in the survey would be adrenal cortical tumors. Feldman [12] regarded ten adrenal tumors reported among his 40 sheep tumors as being of cortical origin. However, only five of our 14 adrenal tumors were diagnosed as adrenal cortical tumors (table 2).

The three adrenal cortical carcinomas and two adenomas found in our survey were not as distinctive in their microscopic appearance as the bovine cortical tumors. The neoplastic cells of the tumors were characteristically arranged in cellular masses and cystic changes and the formation of fibrous trabeculae and small calcified bodies were minimal as compared to corresponding changes in a typical bovine cortical tumor. In the three carcinomas, neoplastic polygonal cells of the tumor were sometimes two or three times the size of normal cortical cells and had large hyperchromatic nuclei and appreciable numbers of mitotic figures.

The more anaplastic appearance of sheep cortical carcinomas, as compared to those observed in cattle in the survey, could be better appreciated with the observation of metastases in two of our animals. One had a renal lymph node metastasis and the other a renal lymph node metastasis and multiple metastases in both lungs. It was of interest that zones of pulmonary adenomatosis were intermixed into many of the lung metastases.

The ovine cortical tumors were all unilateral growths and were firm, grey, lobulated tumors. All except the largest tumor, an adenoma (13 x 8 x 8 cm.) in a lamb, were found in old ewes. This tumor had an unusually benign appearance for a large tumor in a young animal. The three carcinomas were 5 cm. and the other adenoma was 4 cm. in greatest diameters.

Squamous Cell Carcinomas.—The four squamous cell carcinomas were large tumors in old ewes. Only the extensive carcinoma of the vulva (9 x 5 x 1 cm.) had metastases. These metastases were regional in the rectal lymph nodes.

One of the squamous cell carcinomas of the skin (8 x 6 x 2 cm.) replaced the skin adjacent to the left nostril. It extended to involve the mucosal junction of the nostril. The other carcinoma of the skin was a mass (10 x 10 x 6 cm.) around the base of a horn.

Our fourth carcinoma was a growth (6 x 5 x 2 cm.) involving the conjunctiva of the right lower eyelid and adjacent skin. In 1952, Davis and Shorten [7] had described two cases of carcinoma of the eye of sheep to supplement earlier reports [18, 19] of similar tumors.

Ovine Nonepithelial Tumors

Lymphoid Tumors.—Of the 20 lymphoid tumors, 19 were regarded as lymphosarcomas and only one as a possible lymphoma. The lymphoma was a large, encapsulated, cystic growth (17 x 13 x 12 cm.) in the anterior mediastinum of an aged ewe and, perhaps, should be called a malignant tumor on the basis of its size. However, in appearance, it resembled a well-differentiated lymphocytoma and there were no indications in our sections of mitotic activity or anaplasia. The only other solitary lymphoid tumors were large subcutaneous growths which may have arisen in skeletal lymph nodes but at necropsy were invading muscle and connective tissue; one (22 cm., dia.) involved the thigh and the other (12 cm., dia.) the abdominal wall.

The histological morphology of the 19 ovine lymphosarcomas made them easier to divide into sub-groups than the bovine lymphoid tumors. They were classified as seven reticulum cell sarcomas, three follicular lymphomas, five lymphoblastomas, and four lymphocytomas. The follicular lymphomas were the most benign appearing of the four groups but the distribution of lesions in several lymph nodes and one visceral organ in each case (spleen, kidney, and lung, respectively) and the tendency of portions of some of the growths to appear as a diffuse lymphocytoma type of growth with invasive tendencies convinced us that they were malignant tumors. The only tumor in the survey group with lesions still confined within lymph nodes was a lymphocytoma which had at least 15 tumefied skeletal or visceral nodes in different regional anatomic sites.

As in the bovine lymphoid tumors, the most common site of involvement was the combined sublumbar, renal, and internal iliac group of lymph nodes with variable extensions into adjacent tissues from them. Of the 20 cases, 12 had lesions in this area in addition to at least one other lesion in some other anatomic site.

Only 2 of the sheep had cervical lesions comparable to those observed in cattle. There was 1 animal with heart lesions and none with involvement of the stomach. Subcutaneous lesions, however, were more common than in cattle. Massive, subcutaneous, invasive lesions, perhaps originating in lymph nodes, were found in 6 sheep; 2 of the abdomen, 2 of the shoulder, 1 of the thigh, and 1 of the back.

There was splenic involvement in 5 animals. Only 1 sheep had lesions in the lung, liver, and kidney, 2 had multiple lesions in the lungs but no involvement of the kidney, and 3 had liver lesions with no recognizable tumors in the lungs.

Of the 20 sheep with lymphoid tumors, 1 was 1 year old and of unknown sex and 19 were ewes; 3 were 5 years old and the remainder were listed as old.

Myeloid Tumors.—Myeloid tumors are not too uncommon in sheep and two found in this survey in old ewes were malignant. Neither of the tumors exhibited a greenish color on sections of individual growths. When present, this coloration is helpful in making a diagnosis. One of the cases was represented by a solid, greyish, irregularly shaped, infiltrating mass (8 x 2 x 2 cm.) beneath the junction of the costal and diaphragmatic pleura of one pleural cavity. It had produced adhesions to but had not invaded the parenchyma of one lung. On histological section, the tumor was invading the muscles of the ribs and diaphragm. No tumefactions of the bone or bone marrow could be demonstrated.

The other malignant myeloma had multiple lesions in the liver and both cornua of the uterus. Growths were also present in the broad ligament, peritoneum, along the posterior aorta and vena cava, and as large masses which replaced the sublumber, internal iliac, and renal lymph nodes and one external iliac lymph node.

Adrenal Medullary Tumors.—The nine adrenal medullary tumors were remarkably similar in their gross and microscopic appearance and were diagnosed as pheochromocytomas. Only the three largest were regarded as malignant on the basis of their size and invasiveness described in the next paragraph. The tumors had been fixed in chromate salts for special study and only a small proportion of the neoplastic cells contained chromaffin granules. As with similar bovine tumors, the tumors were more representative of growths not differentiated sufficiently to form large numbers of granules.

These tumors were firm, greyish, lobulated, and thinly encapsulated and could not be differentiated with certainty from the adrenal cortical tumors on the basis of their appearance at necropsy. The tumors were too large to have a recognizable point of origin in the remnants of tumefied adrenals. All of the tumors were unilateral growths and were found in old ewes. They varied in size from 1 to 12 cm. in diameter. The two largest pheochromocytomas (12 and 10 cm. in their greatest diameter) were found adhered to the vena cava with lobular masses protruding through the wall into the lumen. The third largest pheochromocytoma (7 cm., dia.) was adhered to a greatly thinned aorta and it too had penetrated as a protruding pedunculated growth into the lumen. We described similar adhesions and penetrations of the aorta as secondary phenomena in our discussions of bovine adrenal tumors.

Leiomyoma.—The only leiomyoma involved the approximate middle segment of the ileum of an old ram. It was an oval, encapsulated growth (13 x 11 x 5 cm.) embedded in the outer musculature of the intestinal wall. On section, the tumor was firm, grey, and finely lobulated. Histologically, interlacing neoplastic smooth muscle cells were quite benign in their appearance. At the junction of the tumor and the muscle, there were many large blood vessels; no neoplastic cells were seen invading this area.

Angioid Tumors.—The angioid tumors were represented by an angioma of the spleen, an angioma of the liver, and an angiosarcoma of the liver. The angioma of the spleen was found in a 6-month-old male lamb and was similar in appearance to the bovine angioid tumors of the spleen. It was a reddish mass (13 x 11 x 5 cm.) with solid and cystic areas and replaced much of the hilus and dorsal border of the spleen.

The angioma of the liver was a solitary, encapsulated, nodular, calcified, and cystic mass (8 x 6 x 4 cm.) in the ventral lobe of the liver of an old ewe. A firm tumor, considered to be an angiosarcoma (10 x 4 x 3 cm.), was found in a 1-year-old lamb. It was red and many small satellite growths adjacent to the main tumor were indicative of the invasiveness of the tumor.

Fibrosarcomas.—Both of the fibrosarcomas were locally invasive and one was metastatic. The metastatic fibrosarcoma was found in an 8-month-old lamb and the primary tumor was a grey, lobulated, subcutaneous mass (15 x 10 x 8 cm.). It was situated in the perianal region with one of its borders attached to the coccygeal vertebrae. Some of the sacral, rectal, and sublumbar, and both of the internal iliac lymph nodes, were enlarged and contained tumor metastases. Mucinous and cystic areas were found in the primary tumor and its metastases.

The other fibrosarcoma was unusual. Nearly two thirds of the pericardium of an old ewe was replaced by a fibrous, cystic, partially calcified tumor (15 x 6 x 6 cm.). The growth was invading adjacent mediastinal tissues and had formed adhesions with the epicardium of the heart.

Chondrosarcomas.—Tumors of cartilage occur much more frequently in sheep than in cattle or swine. (We have no examples in our files of cartilaginous tumors in swine and the only one in cattle is a small, benign chondroma arising in the bronchus of a 2-year-old cow.) In addition to the three chondrosarcomas reported here, we have studied five other ovine cartilaginous tumors. One of the five was a benign solitary growth of the humerus, composed of mature-appearing neoplastic cartilage cells; three were primary chondrosarcomas of the ribs, each with metastases; and one was a solitary chondrosarcoma of the scapula which was quite similar to the one seen in the survey.

The three tumors of cartilage encountered in this survey were regarded as chondrosarcomas be-

cause their anaplastic appearance and invasiveness of bone and bone marrow made them indistinguishable from the three mentioned in the preceding paragraph which had metastasized. They were found in old ewes. The tumors involved the scapula (12 x 7 x 6 cm.), the sternum, and the sternal ends of the first four pair of ribs (16 x 9 x 8 cm.) and the radio-carpal-metacarpal joint (15 x 13 x 10 cm.).

Lipoid Tumors.—The two tumors of neoplastic fat cells were a lipoma of the anterior mediastinum of an old ewe and a liposarcoma of the subcutaneous tissue of the neck in a 4-year-old ram.

The liposarcoma was a massive tumor (16 x 13 x 10 cm.) in the subcutis extending from the angle of the lower jaw to the shoulder and, grossly, could be seen infiltrating through the fascia and between bundles of muscle. Histologically, the tumor appeared invasive and contained immature lipoblasts that showed all stages of vacuolation and blended into small groups of embryonic mesenchymal cells. In contrast to the liposarcoma, the lipoma was an oval, encapsulated growth (6 x 3 x 2 cm.) of mature fat cells in a delicate reticular stroma.

PORCINE TUMORS

Embryonal Nephromas.—Embryonal nephromas were the most frequently diagnosed porcine tumor in the survey. All could be established at necropsy as having their origin in the parenchyma of the kidney. Of the 13 survey animals, 2 were represented by bilateral involvement of the kidney. Multiple lesions were seen in only three affected kidneys and two of these had only two lesions. One of the latter was found in an animal with bilateral involvement.

As observed by others, the tumors varied greatly in size. Single lesions were between 4 and 40 cm. in their greatest diameter; the two largest weighed 43 and 38 lb. All of the affected swine were estimated to be between 6 and 12 months old, and none had metastases. Feldman [3] reported metastases in three of his group of 46 porcine embryonal nephromas. Lung, liver, and lymph node metastases were seen in an embryonal nephroma in tissues from a 3-year-old sow submitted to this laboratory for diagnosis.

Embryonal Sarcoma of Vagina.—The single embryonal sarcoma of the vagina was a tumor covered by an intact stratified epithelium. The bulky folds of the tumefied vagina (fig. 32) were produced by a proliferation of primitive-appearing mesenchymal cells in an edematous, vascular, and fibrous connective tissue stroma. The tumor extended to involve the cervix and body of the uterus but not the vulva or uterine cornua.

This tumor was found in a 6-month-old gilt. An almost identical tumor, from a 1-year-old sow, had been submitted in 1950 for diagnosis.

These two tumors had some resemblance to the "grape-like" or "botryoid" sarcomas of the vagina of children. It is of interest that muscle cells with cross striations are sometimes described as being a component of the human tumors. No striated muscle elements were seen in the two porcine tumors.

Illustrated Case

Figure 32.—Embryonal sarcoma of the vagina of a 6-month-old gilt is shown (case D 3094).

Necropsy.—The distal tubular portion of the genital tract was greatly enlarged. On incision, the vulva appeared normal but the vagina, cervix, and body of the uterus were continuous as a single, enlarged, and hyperplastic structure measuring 45 cm. in length and 15 cm. around the greatest circumference of the vagina. The mucosa was arranged externally as rounded or annular folds which often projected 2 or 3 cm. but retained some resemblance to

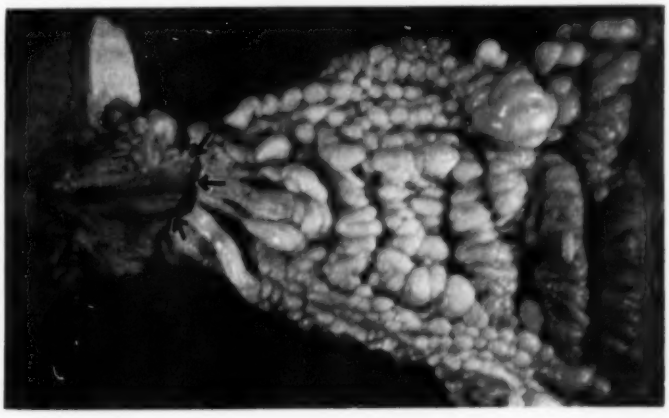


Fig. 32—The rounded or annular folds of the mucosa of the tumefied vagina seen in an embryonal sarcoma of the vagina in a 6-month-old gilt. Arrows indicate the junction of the growth with a normal-appearing vulva (case D 3094).

the normal linear and annular folds of the porcine vagina. On cross section, a papillary or polyp-like arrangement of the tumefied vaginal wall was evident. The submucosal and muscular layers were noticeably increased in thickness with a prominent firm, grey, and fibrous, or soft and mucinoid tissue replacing the normally loose submucosal tissues. The uterine horns and ovaries appeared normal. Hyperplastic changes but no metastases were seen in some of the internal iliac, sublumbar, and rectal lymph nodes.

Lymphoid Tumors.—The nine lymphoid tumors were all diagnosed as lymphosarcomas. One was a follicular lymphoma, and the remainder could be divided into four lymphocytomas and four lymphoblastomas. As in the bovine tumors, several had a cell morphology that was almost between the lymphoblastomas and lymphocytomas and this made classification into groups of doubtful value.

No solitary lymphoid growths were encountered. Two of the hogs had cervical lesions. In two of the nine lymphosarcomas, lesions were recognized only in lymph nodes and tissues adjacent to lymph nodes. These 2 swine were among 6 which had extensive involvement of the combined sublumbar, internal iliac, and renal lymph nodes. The massive subcutaneous neoplastic infiltrations observed in sheep were not seen.

Splenic tumors were found in 5 hogs and liver, but no lung or kidney, growths in 6. The only lesion involving the lung parenchyma was a massive tumor infiltrating the pleural surfaces of the first four ribs in one pleural cavity and producing adhesions and slight invasion of the adjacent apical lobe of the lung. An unusual lesion was a growth (10 cm., dia.) that had replaced much of the bodies of two thoracic vertebrae.

Eight of the 9 swine were estimated to be between 6 and 12 months old and 1 was a 3-year-old sow.

Ganglioneuroma.—The single ganglioneuroma in a strict sense could be called a ganglioneuroblastoma as, histologically, there were a few nerve cells intermediate between neuroblasts and well-differentiated ganglion cells scattered throughout the growth. However, the predominance of mature ganglion cells in a connective tissue stroma which contained many nonmedullated nerve fibers and Schwann's cells and lack of metastases or definite areas of expansive neuroblastic growth favored a diagnosis of ganglioneuroma.

This fibrous, grey, irregularly lobulated tumor (15 x 5 x 5 cm.) was found in a 10-month-old barrow. It was believed to have originated in the celiac plexus or celiaco-mesenteric ganglia as it was firmly attached to the dorsal wall of the abdominal cavity and adhered to the borders of the liver, pancreas, spleen, and diaphragm.

Fibroma.—The fibroma was a firm, encapsulated, subcutaneous growth (3 x 3 x 2 cm.) in the region of the patella. Small, cystic areas containing intercellular mucin were scattered throughout this tumor.

Melanomas.—No malignant and only three benign melanomas were diagnosed in this survey. They were represented by solitary, cutaneous, pigmented tumors of the jowl, flank, and hind leg of swine estimated as 6 to 12 months old. The tumors were oval, encapsulated growths and were 3 to 5 cm. in diameter. Histologically, encapsulated and scattered groups of melanoblasts were seen in each tumor. There was an associated melanosis of regional lymph nodes in each of the animals represented by a deposit of pigment in phagocytic cells or intercellularly as a free pigment. No tumor cells could be detected in these lymph nodes. This pigmentation of regional or distant lymph nodes and, less frequently, of visceral organs can be and is more commonly related to a benign melanosis of the skin. It can often be accurately predicted to be melanosis at necropsy if the associated secondary tissue reaction is minimal.

Melanosis of the skin did complicate the study of porcine melanomas. Undoubtedly some benign melanomas were overlooked in this survey. Feldman's concept [18] that nonelevated, pigmented, cutaneous areas can usually be considered as melanosis is now well established. However, difficulties in differential diagnosis between melanomas and melanosis of the skin were encountered in the examination of pigmented, raised, or pedunculated masses. A histological diagnosis of melanosis was made when encapsulated, free, or phagocytized pigment but not melanoblasts was seen in these lesions.

Only five examples of metastatic malignant melanomas are in our laboratory files and it is concluded that malignant melanomas are not frequently encountered in hogs submitted for slaughter. We have diagnosed no cases of amelanotic melanomas in swine.

DISCUSSION

Variations in the occurrence of tumors at Denver abattoirs have been discussed. An arithmetical percentage of recognized occurrence for each type of tumor over a period of one or two years is seen by comparing tables 1, 2, and 3 with table 5. The collection of bovine eye tumors was not complete so a range of incidence for these tumors would have to be made by using available meat inspection records on condemnations for epithelioma of the eye. Since benign tumors of the eye would not be included with the epitheliomas, there would actually be an even higher occurrence for the bovine ocular squamous cell tumors. Meat inspection regulations indicate that only malignant squamous tumors

of the eye should be reported as epitheliomas. A subsequent article [27] reviews the criteria by which a diagnosis of squamous cell carcinoma of the eye was made in 471 of the 722 squamous cell eye tumors reported in this survey.

The economic loss associated with tumors in cattle, sheep and swine has recently been reviewed in detail by Steiner and Bengston.[47] It is worthwhile to emphasize that the loss from the ocular squamous cell tumors exceeds the combined totals for all other bovine tumors in the United States (table 6). The economic importance of the lymphoid tumors is also shown in this table. One wonders what percentage of the cattle carcasses condemned for carcinoma may not have had metastatic uterine carcinomas. The heretofore little appreciated loss from bovine uterine carcinomas as it relates to periods of nonproductivity and debilitation, as well as death or condemnation in abattoirs, is worthy of serious consideration.

It is more the purpose of this paper to review the common tumors of food-producing animals which might be encountered by meat inspectors, clinicians, or veterinarians engaged in institutional work. Pertinent observations on each type of tumor were included primarily to aid in establishing the identity of the tumors. A veterinarian in general practice will probably encounter representatives of only a few of these groups of tumors.

Ocular squamous cell tumors in cattle are seen frequently and uncomplicated cases are easily diagnosed. Likewise, some of the lymphoid or closely related myeloid tumors do not present too great a problem in diagnosis when there is involvement of the skeletal lymph nodes and subcutaneous tissues. The lymphoid group of tumors is of particular interest because of their frequent occurrence and economic importance in cattle, sheep, and swine; no other important type of tumor is commonly seen in all three species. Most of the skin and subcutaneous neoplasms and the tumors found in accessible portions of body orifices could be diagnosed by biopsy techniques or after surgical extirpation. Many ovarian tumors in cattle and some of the other internal tumors can be either tentatively or positively diagnosed by a careful physical examination.

We had expected a much higher occurrence of liver cell tumors, granulosa cell tumors of the ovary, and epithelial lung tumors in cattle. Granulosa cell tumors particularly are frequently diagnosed clinically and later confirmed histologically.

The high occurrence of Schwann's cell tumors in cattle seems to have been largely overlooked in past reports and surveys of bovine tumors. One may wonder if they have not occurred more frequently in the last two decades. If a genetic factor is related to an increased occurrence, as it is in at least some of the closely related Schwann's cell tumors of man, the problem would be of immediate concern to the geneticist and breeder as well as to the veterinarian.

In the observations made for each type of tumor in the survey, the difficulty of predicting the probability of future metastases of the nonmetastatic tumors has been only briefly discussed. The only logical method of predicting a future metastasis is to correlate the appearance of metastatic tumors with the different development stages, variable degrees of differentiation, or other distinctive characteristics of the nonmetastatic tumor. Obviously, as a tumor collection becomes larger and more representative, the predictability of metastases should also become more accurate.

TABLE 6—The Occurrence* of Tumors in Three Species of Animals Slaughtered at all Federally Inspected Abattoirs in 1953

Species	Number of animals inspected	Number of carcasses condemned at ante or postmortem inspection					Number of parts of carcasses condemned at postmortem inspection		
		Carcinoma	Epithelioma	Leukemia or lymphoma	Sarcoma	Misc.	Carcinoma	Epithelioma	Misc.
Cattle	15,208,023	1,538	4,402	1,518	540	2,247	40	25,608	87
Calves	6,027,449	14	2	100	15	89	—	64	5
Sheep and lambs	13,623,394	51	4	34	26	131	—	—	2
Swine	57,395,484	241	—	529	234	624	—	3	13

* Compiled from the annual report of the Chief of the Bureau of Animal Industry, U. S. Department of Agriculture; — no condemnations.

The experience of other investigators, reference cases in our files, and completed studies of groups of tumors, [28, 29] did influence us appreciably in making a diagnosis of malignancy in individual cases. The same is true for benign tumors. If a tumor was similar in appearance to a large group which seldom or never metastasized, there would be a basis to regard it as a benign growth.

Types of tumors which consistently compress or invade vital organs, or structures, or interfere with function, but seldom metastasize, involve a different consideration in respect to malignancy in a clinical prognosis and also in meat inspection than do tumors with widespread metastases. In addition, size, local invasiveness, rate of growth, and degenerative changes within the tumor can not be completely ignored in a diagnosis of malignancy. There would seem to be value in designating malignant tumors as (1) metastatic, (2) apt to metastasize, (3) locally malignant, and (4) both apt to metastasize and locally malignant.

While many types of tumors predominantly occur in older animals, several groups may appear spontaneously at any age. There should be a general awareness of the groups of tumors which should not be regarded as oddities when they appear in young animals. The survey would indicate that granulosa cell tumors of the ovary, cystadenomas of the gallbladder, lymphoid tumors, and angioid tumors were the tumors encountered most frequently in the younger cattle. Liver cell tumors in sheep, and lymphoid tumors and embryonal nephromas in swine also seemed to occur more often in younger animals than other types of tumors. Malignant melanomas in swine, as previously mentioned, appear most frequently before they reach market age. These statements should not be misconstrued to indicate that these tumors occur more frequently in younger animals as there are many more animals slaughtered in the younger age group. Cancer is predominantly a disease of the older animal.

The scarcity of epithelial tumors in swine is emphasized by our failure to observe a single case in the two-year survey (table 3). Feldman [13] observed only three carcinomas in 76 porcine tumors; they were primary carcinomas of the mammary gland, liver, and skin. Davis, Leeper, and Shelton [8] reported a carcinoma of the adrenal gland among 26 tumors and Jackson [19] described a multiple papilloma of the glottis and two adenomas of the liver.

In the files of this Denver laboratory, there are tissues from seven epithelial porcine tumors. These include two adenocarcinomas of the pancreas with metastases, one adenocarcinoma of the kidney with metastases, and an adenoma of the colon. The other three were squamous cell carcinomas; one was a primary carcinoma of the skin, and two were incomplete cases. A metastatic lesion of the rib was the only tissue in one of these two cases and multiple metastases in the liver represented the submitted lesions in the other.

The importance of conducting surveys on the occurrence of tumors in slaughtered food-producing animals over one or more complete calendar years has been indicated. We believe that in the two years of the survey, examples of all important types of tumors which might be found in animals slaughtered in Denver abattoirs were collected.

SUMMARY

In a 2-year survey of tumors occurring in cattle, sheep, and swine, submitted for slaughter at Denver's federally inspected abattoirs, 1,002 tumors were collected; 908 bovine, 66 ovine, and 28 porcine tumors.

Of the 908 bovine tumors, 722 were squamous cell neoplasms of the eye and were found in 532 cattle during the first year of the survey. The collection of eye tumors was limited to those found in selected abattoirs during one year; their frequent occurrence made them the most important neoplasm encountered in cattle. A diagnosis of squamous cell carcinoma was made in 471 of the 722 eye tumors.

The comparative frequent occurrence of adenocarcinomas of the uterus and lymphosarcomas in cattle in this survey and their predictable early fatal termination made them both economically important. Schwann's cell and adrenal cortical tumors were common bovine tumors with a relatively benign behavior. Liver cell tumors, granulosa cell tumors of the ovary, and epithelial lung tumors of cattle were not found as frequently as had been anticipated.

Neoplastic diseases in slaughtered sheep and swine are much less important than in cattle. Epithelial tumors were equal in occurrence to the nonepithelial tumors in

sheep but were not found in swine in the survey. Lymphosarcomas would appear to be the tumor of the greatest economic importance in both sheep and swine. Embryonal nephromas were the most common tumor diagnosed in swine.

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Some Effects of Chlorpromazine Hydrochloride in Horses

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CHLORPROMAZINE hydrochloride, a relatively new drug, is being widely employed in human medicine for numerous clinical conditions. Therefore, it seemed desirable to conduct certain studies of its effect on animals. The report presented here is based upon preliminary experiments on horses for the purpose of determining a suitable dose range for chlorpromazine and observing its effect on behavior and certain functions in this animal.

Chlorpromazine hydrochloride, known in the United States as thorazine® and in Europe as largactil,® is 10-(3-dimethylaminopropyl)-2-chlorophenothiazine hydrochloride. Chemically, it is closely related to phenothiazine and to the antihistaminic phenargan.® However, it possesses little parasiticidal or antihistamine action.

Chlorpromazine may be classed as a central nervous system depressant and experiments by Courvoisier *et al.*[1] have shown that it produces a significant depression of motor activity. The same workers reported that it enhanced the activity of a number of analgesic drugs and drugs depressing the central nervous system and indicated that it exhibited anticmetic, antipyretic, gangliolytic, and adrenolytic properties. One of the most pronounced effects of the drug is its anticmetic action. Experiments have shown that it will effectively block the emesis produced in dogs by morphine and apomorphine. Doses larger than those needed to inhibit emesis in dogs are reported to produce depression and ataxia lasting for 24 to 48 hours.[2] In studies on anesthetized dogs, Moyer *et al.*[3] found that intravenous doses of 10.0 mg. per kilogram of body weight resulted in a fall of blood pressure, a tachycardia and, in some cases, a decreased cardiac output. Some of the dogs exhibited a moderate diuresis associated with an increased excretion of sodium.

There appears to be general agreement that chlorpromazine can control psychomotor activity in man, and it has been successfully employed in the treatment of various neuropsychiatric disorders.[4-6] Some other uses are to potentiate the action of analgesics and sedatives and to control vomiting in patients with malignancies.[5, 6]

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METHODS

A total of 65 experiments were carried out on 6 horses. The animals used were as follows:

Horse (No.)	Age (yrs.)	Weight (kg.)	Sex
1	12	569	Gelding
2	10	393	Mare
3	9	477	Mare
4	12	464	Mare
5	10	599	Gelding
6	7	553	Gelding

All animals were in good physical condition during the six-month observation.

The experiments performed may be grouped as follows:

Control Experiments.—At least one control experiment was carried out on each animal. During these trials, the horses were confined in slip stalls and observations were made on heart rate and breathing rate at 15-minute intervals for four hours. Thoracic and abdominal breathing patterns were recorded on a smoked-paper kymograph by means of a tambour connected to pneumographs placed at the sixth and seventeenth ribs. Body temperature was recorded at the beginning and at the end of each experiment. One horse had previously been prepared with an exteriorized carotid artery, and blood pressure determinations were made at 15-minute intervals on this animal, using a blood pressure cuff placed about the artery.

Effect of Chlorpromazine Hydrochloride on Heart Rate, Breathing Rate, and Pattern, Blood Pressure, Body Temperature, and Behavior.—A total of 28 trials were carried out on 6 horses confined to slip stalls. Recordings were made on the various functions as described for the control experiments, and the animals were carefully observed for signs of excitement or depression. A 30-minute control period preceded the injection of chlorpromazine hydrochloride, which was given intramuscularly in doses ranging from 0.5 to 4.0 mg. per kilogram of body weight. During the postadministration period, observations were made every ten minutes for one hour, every 20 minutes for the next hour, and every 30 minutes thereafter. The total postadministration period lasted from four to six hours.

Effect of Chlorpromazine Hydrochloride Combined with Meperidine Hydrochloride (Demerol®).*—This group of experiments was conducted in the same manner as those in which only chlorpromazine was given. In six trials, only meperidine hydrochloride was given in doses of 1,000.0 mg.,

* Meperidine employed in these studies was supplied by Winthrop-Stearns Co., New York, N. Y. Demerol is the registered name of Winthrop-Stearns for meperidine hydrochloride.

intramuscularly. In 12 trials, meperidine hydrochloride was given in a dose of either 500.0 or 750.0 mg. and chlorpromazine hydrochloride was injected simultaneously in a dose of 1.5 or 2.0 mg. per kilogram of body wieght.

Effect of Chlorpromazine Hydrochloride on Spontaneous Motor Activity in the Horse.—Ten experiments were performed on 5 horses in which spontaneous motor activity was measured by means of a pedometer ** strapped to the left foreleg so as to record movement. The horses were confined in box stalls for a 48-hour period. The first 24 hours acted as a control period and, at the end of this time, a reading was made from the pedometer and chlorpromazine hydrochloride was injected. At the end of 48 hours, a second reading was made and the activity for the two 24-hour periods was compared.

Effect of Chlorpromazine Hydrochloride on the Blood Picture.— Four experiments were performed on 4 horses which received repeated daily injections of chlorpromazine. A blood sample was taken immediately prior to the first injection of the drug and daily for varying periods following the last injection. One horse received a daily injection of 1.0 mg. per kilogram of body weight for five days and 3 other animals were given 2.0 mg. per kilogram daily for three days.

In addition to the experimental trials, chlorpromazine hydrochloride was also employed in 6 horses which were present as clinical cases in the Veterinary Hospital, University of Pennsylvania.

In all instances, chlorpromazine hydrochloride was injected intramuscularly. The solution for injection was prepared by dissolving the powdered drug in sterile physiological saline solution. In general, a 5 per cent solution was employed.

Meperidine hydrochloride was used in a solution containing 50 mg. per cubic centimeter and was administered intramuscularly.

** Pedometer obtained from New Haven Clock and Watch Co., New Haven, Conn. This instrument was calibrated so as to measure movement in miles and quarter miles.

RESULTS

The effects of chlorpromazine hydrochloride are as follows:

Heart Rate.—There was considerable variation in the effect of chlorpromazine on the heart rate of individual horses (table 1). Following a dose of 2.5 mg. per kilogram body weight, the heart rate increased in five of six trials and the average rate was increased starting at 20 minutes after injection of the drug and lasting for at least four hours. With lower doses, particularly with a dose of 1.5 mg. per kilogram, individual animals showed either an increased or decreased rate and, when compared to control experiments, the average rate was not greatly changed.

Breathing Rate and Pattern.—The effect of chlorpromazine on the breathing rate and pattern was more consistent than its action on heart rate (table 1 and graph 1). In 26 of 28 trials, the breathing rate was

TABLE 1—Effect of Chlorpromazine Hydrochloride on Heart Rate and Breathing Rate of 6 Horses

Dose (mg./kg.)	No. trials	Average change, minutes after chlorpromazine *				
		20	60	120	180	240
		HEART RATE				
None	6	0 (−1 + 3)	0 (− 1 + 4)	+1 (0 + 4)	+1 (− 1 + 3)	0 (− 2 + 2)
0.5	4	+1 (0 + 3)	0 (− 1 + 1)	+1 (− 1 + 7)	0 (− 1 + 3)	−1 (0 − 2)
1.0	4	+2 (−3 + 4)	+2 (− 3 + 4)	+1 (− 3 + 3)	0 (− 2 + 1)	−1 (− 3 + 1)
1.5	6	0 (−8 + 5)	0 (−11 +23)	−2 (−10 +13)	−4 (−13 + 2)	−4 (−10 + 2)
2.0	6	+1 (−5 + 5)	+5 (− 1 + 9)	+2 (− 6 + 6)	+3 (− 6 + 7)	+1 (− 2 + 3)
2.5	6	+5 (0 + 9)	+10 (+ 4 +22)	+9 (− 3 +20)	+9 (− 3 +19)	+5 (− 3 + 9)
		BREATHING RATE				
None	6	+1 (0 + 2)	+1 (− 1 + 3)	+1 (− 1 + 5)	+3 (− 1 + 9)	+1 (0 + 5)
0.5	4	−4 (−1 − 7)	−5 (− 2 − 7)	−6 (− 3 − 8)	−5 (− 2 − 7)	−4 (− 2 − 6)
1.0	4	−2 (0 − 3)	−4 (− 3 − 5)	−4 (− 2 − 5)	−4 (− 3 − 7)	−4 (− 2 − 7)
1.5	6	−6 (−2 −11)	−9 (− 5 −15)	−12 (− 5 −17)	−9 (− 3 −12)	−10 (− 5 −21)
2.0	6	−6 (+2 −17)	−5 (+ 1 −17)	−10 (− 1 −29)	−11 (− 6 −33)	−6 (− 1 − 8)
2.5	6	−9 (+2 −24)	−11 (+ 2 −32)	−11 (+ 2 −31)	−11 (+ 3 −32)	−9 (+ 3 −14)

* Average change in rate per minute compared to control rate before administration of chlorpromazine. Figures in parentheses indicate range.

TABLE 2—Effect of Chlorpromazine Hydrochloride on Blood Pressure of 1 Horse

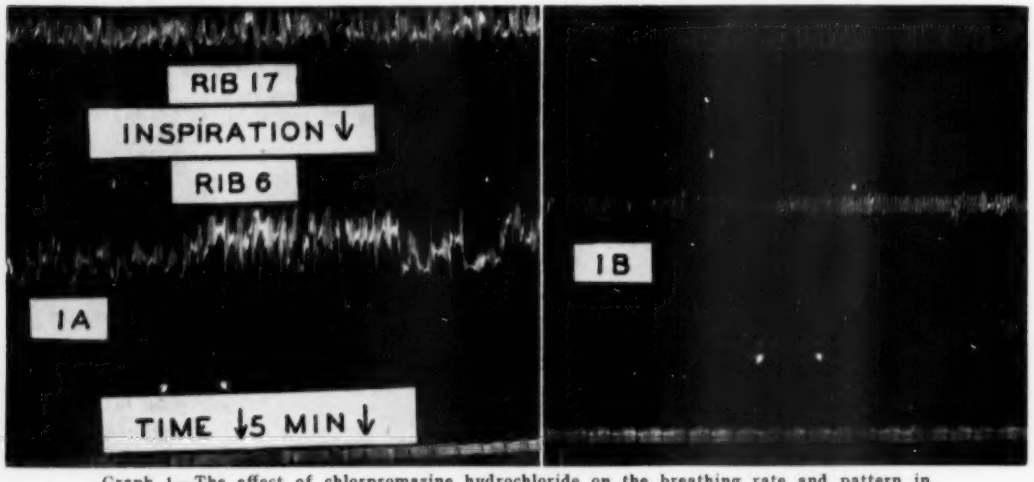
Dose (mg./kg.)	Change in pressure—minutes after chlorpromazine *											
	20		40		60		120		180		240	
	S	D	S	D	S	D	S	D	S	D	S	D
None **	0	0	0	+3	0	+3	+ 6	+ 3	+ 6	+ 3	.....	.....
0.5	.....		.....		−5	+5	−15	+ 5	−15	+ 5	−20	+ 5
1.0	+ 8	0	+ 5	0	0	0	− 5	− 5	+15	+10	+15	+10
2.0 **	− 5	0	−10	−5	− 5	0	−10	0	−15	0	−10	0
2.5 **	−10	−5	−15	−5	−15	−5	−25	−10	−20	−10	−20	−10

* Change in millimeters of Hg. of systolic (S) and diastolic (D) pressure as compared to control values before administration of chlorpromazine; ** average value for two trials.

slowed starting within 20 minutes after injecting the drug. Although there were individual differences in the degree of slowing, this effect of chlorpromazine appeared with doses as low as 0.5 mg. per kilogram. In some instances, a slower rate was still present 24 hours later. In control experiments, the rate either increased or decreased slightly.

All horses showed an irregular or cyclic type of breathing pattern prior to the administration of chlorpromazine. This was evident in recordings from both the thoracic and abdominal pneumographs. Within approximately 20 minutes after injection of the drug, breathing became more regular and deeper. These effects were produced by doses of 1.0 mg. per kilogram of body weight and larger, and usually persisted for at least four hours. In control experiments, the irregular or cyclic type of breathing was present during the entire observation.

Blood Pressure. — Six trials were conducted on 1 horse to observe the effect of chlorpromazine hydrochloride on blood pressure (table 2). This animal had previously been prepared with an exteriorized carotid artery. While a dose of 0.5 mg. per kilogram of body weight resulted in some fall in systolic pressure, consistent effects were not observed until doses of 2.0 or 2.5 mg. per kilogram were given. With the largest dose, a decrease in both systolic and diastolic pressure occurred within 20 minutes and was still present four hours later. From these few experiments, it would appear that sufficient



Graph 1—The effect of chlorpromazine hydrochloride on the breathing rate and pattern in the horse.

A—The record obtained before administration of the drug. Note the irregular, cyclic type of breathing.

B—The record obtained 30 minutes after administration of 2.0 mg. per kilogram of chlorpromazine hydrochloride. Notice the more regular type of breathing and the slower rate. In both records, the upper tracing is from a pneumograph at the seventeenth rib and the lower tracing from a pneumograph at the sixth rib.

doses of chlorpromazine will result in a moderate degree of hypotension in the horse. However, since all of the trials were made on a single animal, further work on other horses is necessary to verify this finding.

TABLE 3—Effect of Chlorpromazine on Spontaneous Motor Activity as Indicated by a Pedometer

Animal	Before drug *	After drug *
Gelding 1	2.50	5.05
	2.25	5.25
	2.50	1.50
Mare 2	2.75	2.10
	1.00	0.60
Mare 3	3.00	2.85
	1.75	2.35
Mare 4	2.00	1.75
	2.75	2.00
Gelding 6	2.75	5.25

* Activity during a 24-hour period, measured in miles. All animals received chlorpromazine (2.0 mg./kg.) intramuscularly.

Body Temperature.—In all experiments, the rectal temperature decreased within the first hour after administering chlorpromazine and remained lower than the control value for at least four hours. In most instances the fall was less than 1.0 F., but in two trials a dose of 2.5 mg. per kilogram of body weight resulted in decreases of 2.2 and 3.0 F., respectively.

Spontaneous Motor Activity.—In the ten trials in which spontaneous activity was measured by means of a pedometer, chlorpromazine hydrochloride was administered in a dose of 2.0 mg. per kilogram of body weight. In six trials, there was a decrease in activity during the 24-hour period following the drug while in the other four trials activity increased (table 3). In those instances in which the activity increased following the drug, the change was of greater magnitude than when it decreased.

Blood Picture.—In the four trials in which chlorpromazine was given by repeated daily injections over a three- or five-day period, there was a decrease in the number of red blood cells and a fall in hemoglobin concentration (table 4). In horse No. 4 it was found that the red blood cell count and hemoglobin concentration did not return to control levels until 13 days after the last injection of the drug. Neither the total number of circulating white blood cells nor the differential count showed any consistent changes in these trials.

Behavior.—Observations on behavior were made on horses confined in slip stalls. The animals had been trained previously to stand quietly in the stalls while an experiment was in progress. With doses of 0.5 mg. per kilogram of body weight the horse remained quiet but showed no signs of depression. Doses of 1.0, 1.5, and 2.0 mg. per kilogram produced depression in some animals but not consistently. In five of the 15 trials in which these doses were employed, there was some evidence of restlessness or excitement. With doses of 2.5, 3.0, or 4.0 mg. per kilogram, signs of depression always appeared. These were usually evident within the first hour after injection of chlorpromazine and, in some, they appeared in as short a time as 20 minutes. The degree of depression tended to increase during the first and second hour after administration of the drug and the animals remained depressed for periods up to six hours and, in a few cases, were still in a depressed state 24 or 48 hours later. In general, horses depressed with chlorpromazine appeared as follows: the neck was extended and the head hung in a position close to the floor. The eyes were partly closed and the animal assumed a dull, sleepy appearance. It had a tendency to spread the hindlegs widely and the front legs stiffly. While in this posi-

TABLE 4—Effect of Chlorpromazine on Red Blood Cells and Hemoglobin of Horses

Horse (No.)	Daily dose (mg./kg.)	Length of treatment (days)	r.b.c. (millions/cmm.)		Hb. (Gm./100 cc.)	
			Before *	After **	Before *	After **
1	1.0	5	8.25	4.00	12.3	9.5
2	2.0	3	8.11	4.76	10.8	8.7
3	2.0	3	8.02	4.60	12.6	8.0
4	2.0	5	6.56	4.13	11.9	8.7

* Preinjection sample taken immediately before first dose of chlorpromazine hydrochloride.
** Postinjection sample in horse 1 taken 48 hr. after last dose of drug; in horses 2, 3 and 4 taken 24 hr. after last dose.

tion the animal often leaned back against the rear wall of the stall and at times appeared on the verge of going down. In most instances, there was definite evidence of ataxia; in making slight movements about the stall the front legs were often crossed and the horse would stagger. The anus was relaxed and in geldings the penis was relaxed and protruding. Despite the appearance of marked depression, the horses could be easily roused by noises or, at irregular intervals, would show some spontaneous recovery. When this occurred, the animal became restless for a short period and would paw or move about the stall. Following these short periods of restlessness, depression would again appear. In two instances, horses went down for short periods. They did not struggle while down and regained their feet without assistance.

Miscellaneous Observations. — Doses of chlorpromazine ranging from 0.5 to 4.0 mg. per kilogram of body weight, had no apparent effect on appetite in horses. Those receiving daily doses for three or five days consumed the usual amount of food and showed no change in the character or frequency of bowel movements.

After injection of the drug, most horses had a slight watery discharge from the nostrils and eyes and passed flatus frequently.

Chlorpromazine was usually injected in a 5 per cent solution and, generally, no more than 10 ml. in any particular site. A slight, localized swelling usually occurred about the site of injection, but subsided within 24 to 48 hours. In one instance, a 10 per cent solution was injected but this produced a large swelling and its use was discontinued.

The effects of chlorpromazine and meperidine are as follows:

Meperidine hydrochloride alone was employed in six trials on 5 horses (table 5). In all cases, an intramuscular dose of 1,000 mg. was used. This dose produced either a slowing or an increase in the heart rate and breathing rate of individual horses, but the average rates were not greatly changed from the control values.

In four trials, chlorpromazine hydrochloride was used in a dose of 1.5 mg. per kilogram of body weight combined with 500 mg. of meperidine hydrochloride. Following this combination, the heart rate either increased or decreased but the average rate for all trials showed little change. The breathing rate was consistently slowed beginning 20 minutes after injection of the drugs and lasting for at least four hours. The degree of slowing was approximately equivalent to that seen when chlorpromazine was administered alone in a dose of 1.5 mg. per kilogram. The breathing pattern became more regular and deeper. Body temperature was lowered in all cases. In three trials, the decrease was less than 1.0 F., but 1 animal showed a fall of 1.4 F. three hours after injection of the drugs. In the one trial in which blood pressure was recorded, both systolic and diastolic pressures showed some decrease. The maximum fall for systolic pressure was 15 mm.

TABLE 5—Effect of Chlorpromazine and Meperidine on Heart Rate and Breathing Rate of 5 Horses

Chlorpromazine (mg./kg.)	Meperidine (mg./kg.)	No. trials	Average change in rate*—minutes after injection				
			20	60	120	180	240
			HEART RATE				
None	1,000	6	0	−1	−3	−2	−1
			(− 2 + 2)	(− 5 + 4)	(− 6 + 5)	(− 6 + 3)	(− 6 + 4)
1.5	500	4	−1	−1	−3	−2	−4
			(− 3 + 1)	(−10 + 6)	(−11 − 1)	(−12 + 1)	(−11 + 2)
1.5	750	4	+3	+5	+4	+1	+2
			(− 4 +10)	(− 2 +12)	(− 6 +12)	(− 6 + 8)	(− 4 + 6)
2.0	500	4	−1	−1	+3	−2	−1
			(− 3 + 2)	(− 4 0)	(− 5 +14)	(− 3 + 5)	(− 4 + 5)
			BREATHING RATE				
None	1,000	6	−1	−1	0	+2	0
			(−10 + 6)	(− 3 + 3)	(− 5 + 7)	(− 2 + 7)	(− 2 + 2)
1.5	500	4	−4	−9	−9	−9	−9
			(0 − 7)	(− 3 −18)	(− 1 −18)	(− 1 −20)	(− 1 −17)
1.5	750	4	−9	−14	−14	−15	−15
			(0 −14)	(− 9 −13)	(−10 −22)	(−11 −22)	(−11 −21)
2.0	500	4	−7	−5	−4	−7	−8
			(− 2 −12)	(0 −13)	(− 4 − 7)	(− 5 −12)	(− 2 −13)

* Average change in rate per minute compared to control rate before administration of the drugs. Figures in parentheses indicate range.

Hg. and for diastolic 5 mm. Hg. Horses receiving this combination of chlorpromazine and meperidine showed slight depression, as previously described when chlorpromazine was used alone.

In four other experiments, chlorpromazine hydrochloride was given in a dose of 1.5 mg. per kilogram of body weight combined with 750 mg. of meperidine hydrochloride. With this combination of drugs, the average heart rate showed little change but individual horses exhibited a variable response, some showing an increased rate and others a decrease. The breathing rate was decreased in all trials and the breathing pattern became deeper and more regular. The other changes were similar to those previously described for this dose of chlorpromazine and the smaller dose of meperidine. However, a greater degree of depression was observed. The animals appeared similar to those receiving a dose of 2.5 mg. per kilogram of chlorpromazine except there were periods in which restlessness was more pronounced. One horse went down for a short time three hours after injection of the drugs.

In four experiments, chlorpromazine hydrochloride was given in a dose of 2.0 mg. per kilogram of body weight and meperidine hydrochloride was given simultaneously in a dose of 500 mg. This combination produced changes in the heart rate and body temperature similar to those described for other combinations of the two drugs, but the breathing rate, while affected less, was slowed in every case. The degree of depression was comparable to that observed when a dose of 2.5 mg. per kilogram of chlorpromazine alone was given. In one experiment, the horse went down briefly approximately two and a half hours after administration of the drugs.

The clinical observations were as follows:

Several opportunities arose to employ chlorpromazine hydrochloride in horses suffering from certain diseases. The object of using the drug in these cases was not necessarily that of evaluating its therapeutic properties, but rather to observe its effects on behavior of horses under clinical conditions.

Case 1.—A Thoroughbred yearling, diagnosed as a "wobbler" (equine ataxia), was given chlorpromazine in a dose of 2.0 mg. per kilogram of body weight. Within 30 minutes, definite signs of depression appeared. Whereas the animal had previously been difficult to approach and catch in a box stall, this was now easily accomplished. Eighty minutes after injection with chlorpromazine the horse went down and did not get up for approximately twelve hours. During this time the animal remained quiet and was able to roll up on its sternum and eat small amounts of hay. Signs of depression continued for 24 hours. There was no apparent improvement of the clinical condition.

Case 2.—This 4-year-old Thoroughbred had a fistulous tract which opened between the mandibles. It was decided to inject the tract with a radiopaque material in order to trace its course by means of radiographs.

Chlorpromazine (2.0 mg./kg. of body weight), administered in an attempt to produce sedation, had little effect.

Case 3.—A 500-kg. horse with a severe chronic lameness and unable to bear weight on the left foreleg was given a dose of chlorpromazine hydrochloride (2.5 mg./kg. of body weight). This dose produced signs of depression and ataxia and the animal was observed to place some weight on the leg as it moved about in a box stall.

Case 4.—This Thoroughbred gelding, presented at the Veterinary Hospital for x-ray therapy, became excited when placed in stocks and sedation was necessary before treatment could be started. Chlorpromazine hydrochloride, in a dose of 2.0 mg. per kilogram, combined with 500 mg. of meperidine hydrochloride did not produce any apparent depression and was of no aid in the management of this animal.

Case 5.—A 950-lb. Tennessee Walking horse, showing lameness in the left foreleg, was given chlorpromazine hydrochloride (2.5 mg./kg. of body weight) combined with 750 mg. of meperidine hydrochloride. Within 30 minutes definite signs of depression appeared, and within 45 minutes ataxia was evident. The animal swayed from side to side and, on walking, would stumble. Signs of depression were still present three hours later when observations were discontinued. No attempt was made to evaluate the drugs' effect on the lameness.

Case 6.—This Thoroughbred mare, with her first foal, had allowed the foal to nurse until the third day when she ob-

jected rather violently and would not allow the foal near her. For the next three days, it was necessary to place a twitch on the mare before the foal could nurse. On the sixth day after foaling, a dose of 1.0 mg. per kilogram of chlorpromazine hydrochloride was administered. Six hours later, the mare allowed the foal to nurse and thereafter showed a normal brood mare-type of behavior toward her offspring.

DISCUSSION

When chlorpromazine hydrochloride is given to horses, it appears to produce many of the same changes observed in dogs and other smaller laboratory animals. These include some degree of tachycardia, a moderate lowering of blood pressure, and a slight fall in body temperature. One of the most consistent and marked effects in horses is the production of a slower, more regular, and deeper breathing pattern. This effect appears with doses lower than those necessary to produce other changes.

Some effects become evident within 20 to 30 minutes after intramuscular injection, indicating that the drug is rapidly absorbed. In general, maximum effects are observed during the first and second hour after injection and are still present at least four to six hours later. The fact that signs of depression and other changes may still be present as long as 24 to 48 hours after a single injection of larger doses of chlorpromazine (2.5 to 4.0 mg./ kg.) probably indicates that the drug is slowly metabolized or eliminated in the horse.

One of the primary objectives of this study was to observe the effects of chlorpromazine on the behavior of horses. Experimental work with laboratory animals and clinical experience in man have shown that the drug exerts a sedative effect. In horses, doses of 2.5 to 4.0 mg. per kilogram were found to produce signs of a moderate to marked depression. A particular feature of this action is that the animals can be aroused easily from a state of deep depression. Even though horses frequently showed signs of ataxia and occasionally went down, they did not struggle while down and were able to regain their feet quickly. These effects are similar to those observed in man, where Winkleman [4] has reported that, although the drug may produce sleep, the patients are easily aroused and on awakening are responsive and coherent.

In interpreting the effects of chlorpromazine on behavior in horses, it must be remembered that only gentle, quiet animals were employed in these studies and that they were confined to stalls during experiments. Whether chlorpromazine will significantly depress horses under clinical conditions is not known. In the few clinical trials in which the drug was given to excitable horses, the results were not encouraging; larger doses may be necessary to depress restless or excitable animals. In several of the clinical cases, the dose employed was 2.0 mg. per kilogram and, on the basis of the experimental trials, it seems that doses of 2.5 mg. per kilogram or higher are needed to consistently depress horses. The pedometer experiments indicated that a dose of 2.0 mg. per kilogram is not sufficient to depress spontaneous motor activity in horses.

Chlorpromazine is reported to enhance the activity of a number of analgesic drugs and of drugs that depress the central nervous system. In man, the effective dosage of sedatives and analgesics may be reduced by as much as one half when they are used in combination with chlorpromazine. Archer [7] has reported that meperidine hydrochloride in doses of 1,000 mg. intramuscularly exerts a sedative effect on horses with colic. In this study, a similar dose failed to produce any observable effect. This difference may be explained by the fact that the drug's sedative effect on horses with colic is probably related to its analgesic action in relieving pain and not to a general depressant action on the central nervous system. In the trials reported here, the horses were not subjected to pain and were not excited.

However, it is of interest that when relatively small doses of the two drugs were used in combination, signs of a moderate to marked depression appeared. The degree of depression was comparable to that seen when larger doses of chlorpromazine were used alone. Thus, it would appear that the drugs in some manner complement one another in their effect on horses. In one clinical trial, the combined drugs produced depression, but more work is needed to determine whether the combination might be of value in quieting excit-

able horses and, particularly, to establish the proper dose of each necessary for optimal effects.

No attempt was made to study the side effects or toxic actions of chlorpromazine. In man, agranulocytosis, dermatitis, jaundice, and the occurrence of parkinsonism have been observed in a small percentage of patients treated.[5] None of these effects was observed in horses, but repeated daily doses of the drug resulted in a decrease in circulating red blood cells and a fall in hemoglobin concentration. In one trial, this effect persisted for two weeks after the last injection of the drug.

Since chlorpromazine and phenothiazine are closely related chemically, it is interesting to speculate on the possibility of this effect being related to the hemolytic anemia sometimes seen in horses treated with that anthelmintic. In any event, further work is indicated to determine whether this is a consistent occurrence in horses receiving chlorpromazine hydrochloride.

The need for improved sedative drugs for horses is well recognized by veterinarians. Sedatives which would consistently depress horses but which would allow the animals to remain in a standing position would be especially useful in a number of situations. These include the shipment of excitable horses and the restraint of animals during such procedures as making radiographs, performing dental work, and in minor surgical procedures. Under certain conditions, such drugs would also be of value as preanesthetic agents prior to general surgery.

The use of meperidine hydrochloride as a sedative in colic has been mentioned but some clinicians report that it will not consistently produce sedation. Raker [6] has found that, occasionally, a dose of 1,000 mg. may actually cause some animals to become more restless.

The results of experimental work on chlorpromazine hydrochloride reported here can not be transferred directly to clinical situations. It consistently produced depression when given in a sufficient dose, but this does not mean that similar doses will result in a desirable type of sedation under clinical conditions. However, critical clinical studies on the drug as a sedative for horses are indicated. The anemia produced by chlorpromazine in the experimental studies reported here may act as a serious objection to the use of the drug in horses and, in any clinical trial, this effect should be studied carefully.

The experience in using chlorpromazine on the mare which refused to nurse her foal suggests other possible uses of the drug in veterinary medicine. Little is known about the possibility of neuropsychiatric disorders in animals but the abnormal behavior of this animal might be explained on this basis. Winkleman [4] has proposed that chlorpromazine may act in man by altering the conscious perception of, and pattern of reaction to, personality-disturbing stimuli. Much more work is needed before one may hope to reach any logical conclusions regarding the possible modes of action of chlorpromazine but the use of it, and other such drugs, may eventually provide answers to some of the complex problems of abnormal behavior in animals.

Summary

In a preliminary study on the pharmacological activity of chlorpromazine hydrochloride in horses, intramuscular doses of 2.0 to 4.0 mg. per kilogram of body weight were found to produce a slight tachycardia and hypotension, a slower, more regular, and deeper breathing pattern, and a slight fall in body temperature.

In horses confined under quiet conditions in slip stalls, doses of 2.5 mg. per kilogram or larger, resulted in signs of moderate to marked depression which started within one hour after injection and lasted for at least six hours. In some instances, the horses were still depressed 24 to 48 hours later.

Chlorpromazine hydrochloride (1.5 or 2.0 mg./kg.) when combined with meperidine hydrochloride (750.0 or 500.0 mg.) produced a degree of depression comparable to that seen with larger doses of chlorpromazine when given alone.

Repeated doses of chlorpromazine hydrochloride for three days (2.0 mg./kg. daily) or five days (1.0 mg./kg. daily) resulted in a fall in the number of circulating red blood cells and hemoglobin concentration. This effect may last for as long as two weeks.

The use of chlorpromazine hydrochloride in several clinical cases is described and the

possibilities for its use as a therapeutic agent are discussed briefly.

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The Effect of Sulfanilamide on Phosphorus Metabolism of Horses

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THE SULFONAMIDES are of great use in veterinary medicine because they are effective following oral administration. Their bacteriostatic effect is well known and is not discussed in this paper. Practice, however, has shown that excessive treatment with sulfonamides may seriously affect domestic animals and cause decreased efficiency and in some cases even death. For this reason, investigations have been carried out on the toxic activity of sulfanilamide.

REVIEW OF THE LITERATURE

From earlier publications,[1,2] it is known that sulfanilamide has markedly different toxic effects on cows, dogs, and horses. Therapeutic doses of sulfanilamide often cause cows in periods of high milk production to become apathetic and depressed, resulting in decreased milk production; these symptoms are frequently accompanied by acetonemia. In experiments with healthy dogs, however, it has been observed that corresponding doses of sulfanilamide do not result in any toxic signs, even if the drug were given continually for as long as six months. Horses react in various ways, some showing allergic signs after a few doses, while others are not affected until after 20 or 30 days of continued treatment. Our present experiments are devoted to the effect of sulfanilamide on the phosphorus metabolism of horses.

METHOD

To investigate whether the phosphorus metabolism was affected to any degree by sulfanilamide, a single dose was given to horses; then, chemical analyses of different phosphorous fractions in the blood were made.

In order to determine whether clinical signs of intoxication are followed by any systemic effect on the metabolism, chemical analyses were made of different phosphorous fractions and of alkaline phosphatase in the blood, and of phosphorus elimination in the urine during continued treatment of 7 horses. The time when clinical signs appeared was recorded.

The toxic effect of sulfanilamide, in experiments with laboratory animals, has been counteracted by para-aminobenzoic acid (Paba).[1,3] Therefore, it was sought to determine whether Paba might also counteract the effect of sulfanilamide on the analysed phosphorous fractions in horses.

The phosphorus analyses in the blood were made according to Fiske-Subbarrow[4] and in the urine according to Briggs.[5] The phosphatase analyses were made according to Bodansky.[6] Hematocrit values were registered after centrifugation for ten minutes at 2,000 r.p.m.

The research animals used were ordinary horses, 4 to 8 years old, and free from any clinical signs of disease. Their feeding schedules and diet were uniform and, except for 1, they were stabled during the experiments.

EXPERIMENT 1, SINGLE DOSE TREATMENT

In seven experiments with horses, 0.25 Gm. of sulfanilamide per kilogram of body weight was given as a single oral dose. Analyses of inorganic phosphorous fractions in heparinized whole blood, plasma, and corpuscles were made. The blood samples were taken before the administration of the drug and three and seven hours after treatment (table 1a).

RESULTS

As the blood samples were collected at different times of the day, some investigations were made on untreated horses to determine if variations might depend upon normal rhythmic metabolic changes. However, statistically significant changes did not occur (table 1b). After the administration of sulfanilamide to horses, there was a statistically significant decrease of inorganic phosphorus in the whole blood and plasma after three hours (table 1a). No similar effect was observed on the inorganic phosphorus of the whole blood when sulfanilamide and Paba were given in the proportion of 100:1 (table 1c). Organic phosphorous fractions, alkaline phosphatase, and the pH values of the blood were also checked when sulfanilamide was administered but these showed no significant change.

Experiment 2, Continued Treatment.—In the following experiments the animals were given daily doses of the drug until they showed signs of intoxication; then

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TABLE 1a—Blood Values After Administration to Horses of 0.25 Gm. of Sulfanilamide per Kilogram of Body Weight

Hour blood samples taken	Whole blood inorganic phosphorus (mg./100 cc.)	Plasma inorganic phosphorus (mg./100 cc.)	Red blood cells inorganic phosphorus (mg./100 cc.)	Alkaline phosphatase (units/100 cc.)
11 a.m. (just before treatment)	2.29 ± 0.08	3.00 ± 0.15	1.15 ± 0.29	7.44 ± 1.22
2 p.m.	1.76 ± 0.12	2.07 ± 0.25	1.45 ± 0.14	7.45 ± 1.11
6 p.m.	2.06 ± 0.19	2.48 ± 0.33	1.38 ± 0.15	7.46 ± 1.38
	n = 7	n = 6	n = 5	n = 5
Difference between values at 11 a.m. and 2 p.m.	t = 3.55*	t = 3.24*	t = 0.95	t = 0

TABLE 1b—Corresponding Blood Values of Phosphorous Fractions in Untreated Horses

11 a.m.	2.43 ± 0.21	2.99 ± 0.17	1.65 ± 0.47	7.47 ± 1.19
2 p.m.	2.31 ± 0.22	2.80 ± 0.22	1.46 ± 0.40	8.78 ± 1.66
6 p.m.	2.33 ± 0.22	2.77 ± 0.22	1.34 ± 0.28	8.90 ± 1.48
	n = 7	n = 6	n = 5	n = 5

TABLE 1c—Inorganic Phosphorous in Whole Blood After Administration of 0.25 Gm. Sulfanilamide and 0.0025 Gm. of Para-aminobenzoic Acid Per Kilogram of Body Weight

11 a.m. (just before treatment)	2.42 ± 0.15	.....	.....	.....
2 p.m.	2.33 ± 0.29	.....	.....	.....
6 p.m.	2.28 ± 0.31	.....	.....	.....
	n = 6	.....	.....	.....

* Statistical significance: the probability of equal chance effect < 0.01; † n = number.

Paba was added to sulfanilamide and the experiment was continued. If the symptoms were severe and endangered the life of the animal, the administration of the drug was stopped. Blood samples were taken and the drug administered at 11:00 a.m.

Case 1 (Table 2).—This 590-kg. (1,300-lb.) horse was given 100 Gm. (3.527 oz.) of sulfanilamide every day for 36 days. On the twenty-first day, slight toxic signs appeared. The treatment, however, was continued for 16 additional days until the toxic action endangered the horse. On the thirty-sixth day and for the following seven days, 1 Gm. of Paba was added to the daily dose of sulfanilamide. The clinical signs disappeared on the thirty-seventh

TABLE 2 (Case 1)—Effect of Sulfanilamide (100 Gm.) Daily for 43 Days, with Para-aminobenzoic Acid (Paba) After 36 Days, on a 590-kg. (1,300-lb.) Horse

Day of experiment	Hematocrit	Whole blood: Inorganic phosphorus (mg./100 cc.)	Whole blood: Ester phosphorus (mg./100 cc.)	Plasma: Inorganic phosphorus (mg./100 cc.)	Plasma: Ester phosphorus (mg./100 cc.)	Red blood cells: Inorganic phosphorus (mg./100 cc.)	Red blood cells: Ester phosphorus (mg./100 cc.)	Alkaline phosphatase (units/100 cc.)	Phosphorus elimination in urine (mg./day)	Clinical symptoms
Normal values without treatment										
1st	23	2.3	14.6	2.7	0.1	0.4	61.4	6.4	51	.....
2nd	23	2.1	14.2	2.7	0.1	0.3	60.2	6.1	46	.....
Dose of 100 Gm. of sulfanilamide given daily										
16th	23	1.4	13.7	1.8	0.9	0.2	56.6	5.7	1,808	.....
21st	26	1.7	11.6	2.0	1.6	0.1	40.0	6.6	.....	Depression.
22nd	28	1.3	12.9	1.6	0.4	0.4	45.1	5.8	.....	Depression.
30th	26	0.8	12.4	1.3	0.8	0.4	45.5	6.0	1,014	Depression.
36th	*Dose of 100 Gm. of sulfanilamide plus 1 Gm. of Paba given daily*									Severe depression.
37th	36	1.9	16.7	2.6	0	0.8	46.4	7.1	.....	End of depression
38th	32	1.6	16.0	2.1	0.3	0.4	49.5	5.8	.....	.....
40th	41	1.5	15.0	2.2	0.6	0.4	36.9	5.7	.....	.....
42nd	43	2.0	18.3	3.8	0.6	0.4	41.4	6.8	51	.....
43rd	34	2.6	14.9	3.2	0.5	0.4	48.5	....	60	.....

	Significance of differences (mean values): Inorganic phosphorus whole blood	Inorganic phosphorus plasma	Urine phosphorus
Between pretreatment and treatment with sulfanilamide	t = 6.861*	t = 4.232	t = 3.414†
Between treatment with sulfanilamide and treatment with sulfanilamide plus Paba	.....	t = 4.163†	.....

* Probability of equal chance effect < 0.05; † probability of equal chance effect < 0.05 > 0.01.

TABLE 3 (Case 2)—Effect of Sulfanilamide (100 Gm.) Daily for 24 Days, with Para-aminobenzoic Acid (Paba) After 20 Days, on a 640-kg. (1,411-lb.) Horse

Day of experiment	Hematocrit	Whole blood: Inorganic phosphorus (mg./100 cc.)	Whole blood: Ester phosphorus (mg./100 cc.)	Plasma: Inorganic phosphorus (mg./100 cc.)	Plasma: Ester phosphorus (mg./100 cc.)	Red blood cells: Inorganic phosphorus (mg./100 cc.)	Red blood cells: Ester phosphorus (mg./100 cc.)	Alkaline phosphatase (units/100 cc.)	Phosphorus elimination in urine (mg./day)	Clinical symptoms
	Normal values without treatment									
.....	27	1.9	14.2	2.5	0.4	0.9	56.2	6.2	56	.....
	Dose of 100 Gm. of sulfanilamide given daily									
15th	24	1.5	13.6	1.7	0.8	0.8	54.2	6.0	....	.....
	Dose of 100 Gm. of sulfanilamide plus 2 Gm. of Paba given daily									
20th	24	1.9	11.3	2.1	1.9	1.4	41.5	5.6	320	Depression; urticaria.
21st	30	2.2	15.6	2.7	....	0.1	.....	4.4	....	Urticaria; edema under abdomen.
	Dose of 100 Gm. of sulfanilamide plus 1 Gm. of Paba given daily									
22nd	37	2.4	19.3	3.7	0	0.2	52.1	5.4	120	Urticaria; edema under abdomen onto forelegs and head.
	No treatment given									
23rd	36	2.3	18.7	3.1	0	0.9	51.8	8.2	....	.....
24th	28	2.2	15.0	2.9	0.2	0.6	53.2	5.5	....	Died.

day and did not appear again for the duration of the experiment, which was completed on the forty-third day.

During the administration of the sulfanilamide, the hematocrit values did not change significantly but increased after adding Paba. Inorganic phosphorus in whole blood and plasma decreased significantly and highly significantly, respectively, during the administration of sulfanilamide and increased correspondingly after adding Paba. Ester phosphorus decreased in whole blood and red blood cells during administration of sulfanilamide but increased in plasma. After adding Paba, there was an increase of ester phosphorus in whole blood. Elimination of phosphorus in urine was greatly increased during the administration of sulfanilamide but decreased again after adding Paba.

Case 2 (Table 3).—This 640-kg. (1,411-lb.) horse suddenly reacted with severe depression and urticaria after 20 daily administrations of 100 Gm. of sulfanilamide; on the afternoon of the twentieth day, 2 Gm. of Paba was given in order to counteract the effect of sulfanilamide. On the twenty-first day, urticaria was still more pronounced and edema appeared under the abdomen. The horse was given the same dose of sulfanilamide and Paba as the day before. The animal then became more lively, but the edema and urticaria did not disappear. On the morning of the twenty-second day, the symptoms had become still

TABLE 4 (Case 3)—Effect of Sulfanilamide (100 Gm.) Daily for 16 Days, with Para-aminobenzoic Acid (Paba) After 15 Days, on a 625-kg. (1,378-lb.) Horse

Day of experiment	Hematocrit	Whole blood: Inorganic phosphorus (mg./100 cc.)	Whole blood: Ester phosphorus (mg./100 cc.)	Plasma: Inorganic phosphorus (mg./100 cc.)	Plasma: Ester phosphorus (mg./100 cc.)	Red blood cells: Inorganic phosphorus (mg./100 cc.)	Red blood cells: Ester phosphorus (mg./100 cc.)	Alkaline phosphatase (units/100 cc.)	Phosphorus elimination in urine (mg./day)	Clinical symptoms
	Normal values without treatment									
1st	35	1.7	15.3	2.5	0.3	1.0	54.0	5.2	77	.....
2nd	36	2.2	19.6	2.4	0.1	0.9	45.5	4.8	76	.....
	Dose of 100 Gm. of sulfanilamide given daily									
2nd	38	2.5	15.0	2.6	0	0.7	39.4	5.6	....	.....
3rd	37	2.2	15.4	2.6	0.8	1.4	40.2	4.9	....	.....
11th	27	1.6	13.7	2.0	0.8	0.6	48.5	4.6	249	Urticaria.
14th	30	1.5	16.8	1.9	1.0	0	53.6	4.2	140	Depression.
	Dose of 100 Gm. of sulfanilamide plus 1 Gm. of Paba given daily									
15th	48	1.8	21.9	3.2	0.2	0.1	45.4	4.7	....	Severe edema in head; colic; icterus.
16th	50	3.4	20.9	6.7	0.3	0.1	41.0	10.2	....	Severe edema; death.

more severe and the edema had spread over the forelegs and head; therefore, the dose of Paba was decreased to 1 Gm. and the next day all treatment was stopped. Blood samples were collected for the following two days, when the horse died. Necropsy showed multiple emboli in the forelegs and head and severe edema under the skin of the forelegs, chest, and head.

The hematocrit values were increased after Paba was added. Inorganic phosphorus in the whole blood increased very slightly when Paba was added. The inorganic phosphorus in plasma decreased under the administration of sulfanilamide and increased under the administration of Paba. Ester phosphorus in the whole blood and in the red blood cells decreased with the administration of sulfanilamide but increased when Paba was administered while, in plasma, it increased with sulfanilamide and decreased with Paba. The elimination of phosphorus in the urine moderately increased with the administration of sulfanilamide and decreased by more than half when Paba was added.

Case 3 (Table 4).—After 11 daily doses of 100 Gm. of sulfanilamide, this 625-kg. (1,378-lb.) horse showed urticaria and, after 14 days, depression. The following day, there were severe symptoms with edema of the head and colic (invaginatie jejunalis). On days 15 and 16, the horse was given 1 Gm. of Paba added to sulfanilamide, but with no recovery; the horse died.

The hematocrit values decreased at the same time that urticaria appeared but increased greatly with the colic. With the administration of sulfanilamide, inorganic phosphorus in the whole blood and plasma decreased but increased when the symptoms became severe. In the red blood cells, there was a decrease. Ester phosphorus in the whole blood and red blood cells temporarily decreased with the administration of sulfanilamide. Ester phosphorus in plasma showed a temporary increase. Phosphorus elimination in urine increased moderately during the administration of sulfanilamide.

Case 4 (Table 5).—From earlier experiments, it was known that this 580-kg. (1,278-lb.) horse was highly sensitive to sulfanilamide. For that reason, the horse was given only 75 Gm. of sulfanilamide in the morning and 50 Gm. in the evening of the first day. As soon as 24 hours, after the first dose, clinical semptoms appeared, with depression and edema under the abdomen and rigidity of the muscles. The following two days, the horse showed more severe symptoms and the daily dose was cut to 50 Gm., then discontinued. The horse recovered and, on the seventh day of the experiment, he was free of symptoms.

The hematocrit values decreased about 5 per cent during the administration of the drug. Inorganic phosphorus in the whole blood and plasma increased at first, then decreased. After the treatment was stopped, it increased again. Ester phos-

TABLE 5 (Case 4)—Effect of Sulfanilamide on a Horse Known to Be Sensitive

Day of experiment	Hematocrit	Whole blood: Inorganic phosphorus (mg./100 cc.)	Whole blood: Ester phosphorus (mg./100 cc.)	Plasma: Inorganic phosphorus (mg./100 cc.)	Plasma: Ester phosphorus (mg./100 cc.)	Red blood cells: Inorganic phosphorus (mg./100 cc.)	Red blood cells: Ester phosphorus (mg./100 cc.)	Alkaline phosphatase (units/100 cc.)	Phosphorus elimination in urine (mg./day)	Clinical symptoms
	Normal values without treatment									
.....	31	1.6	11.2	1.8	0.4	1.0	37.9	5.9	.....	.....
.....	29	1.6	11.3	1.8	0.4	1.1	35.8	6.6	268	.....
.....	28	1.6	10.9	....	0.4	....	37.9	5.9	.....	.....
1st	*Dose of 75 Gm. in the morning plus 50 Gm. in the evening of sulfanilamide given*									
	Dose of 50 Gm. of sulfanilamide given daily									
2nd	24	1.8	12.7	2.5	0.2	0.1	52.2	6.7	2,815	Depression; edema.
3rd	24	1.2	16.9	1.8	0.5	0	68.9	6.4	175	Edema; urticaria; eosinophilic leukocytes in blood, 14%.
	No treatment given									
4th	25	1.8	14.2	2.2	0.6	0.7	54.8	7.3	.....	Continued edema.
5th	25	2.8	10.3	3.4	0.1	0.8	41.3	6.3	.....	.....
6th	25	2.6	10.8	3.4	0.1	0.8	42.8	6.3	.....	.....
7th	26	1.9	11.4	2.5	0.4	0.3	42.7	6.2	.....	Recovered; eosinophilic leukocytes in blood, 2%.

phorus in the whole blood and red blood cells increased during the administration of the drug but, when the horse became better, these values returned to normal. Phosphorus elimination in the urine markedly increased during the first day of treatment but became normal on the second day.

Case 5 (Table 6).—This 650-kg. (1,433-lb.) horse was given 100 Gm. of sulfanilamide for seven days. On the second day, he showed decreased appetite and, on the eighth day, edema under the abdomen and muscle rigidity appeared. Thereafter, 1 Gm. of Paba was added to the daily dose of sulfanilamide. Because of the severe clinical symptoms, the dose of both drugs was decreased by half the following day and continued for three more days. The edema diminished and disappeared during the administration of both drugs.

The hematocrit values decreased during the administration of sulfanilamide. After the experiment was finished, they returned to normal. There was a highly significant decrease in inorganic phosphorus in the whole blood and plasma after sulfanilamide treatment. After adding Paba, inorganic phosphorus in the plasma rose significantly. Ester phosphorus in the whole blood decreased slowly; in the red blood cells, it first increased, then decreased, and increased again after adding Paba. Phosphatase progressively decreased after the addition of sulfanilamide but began to increase again after adding Paba. The elimination of phosphorus in urine approximately doubled after sulfanilamide administration but decreased again when Paba was added.

Case 6 (Table 7).—This 582-kg. (1,283-lb.) horse was ridden daily during the entire experiment and was given 100 Gm. of sulfanilamide daily for 27 days, without

TABLE 6 (Case 5)—Effect of Sulfanilamide (100 Gm.) Daily, with Paba After Seven Days, on a 650-kg. Horse

Day of experiment	Hematocrit	Whole blood		Plasma		Red blood cells		Alkaline phosphatase (units/100 cc.)	Phosphorus elimination in urine (mg./day)	Clinical symptoms
		Inorganic phosphorus (mg./100 cc.)	Ester phosphorus (mg./100 cc.)	Inorganic phosphorus (mg./100 cc.)	Ester phosphorus (mg./100 cc.)	Inorganic phosphorus (mg./100 cc.)	Ester phosphorus (mg./100 cc.)			
Normal values without treatment										
1st	30	1.9	11.5	2.8	....	0	40.0	6.2	184	.....
2nd	27	2.1	12.9	3.1	0.6	0	.....	5.5	160	.....
3rd	28	2.3	.....	3.3	....	0	.....	6.7	.....	.....
Dose of 100 Gm. of sulfanilamide given daily										
2nd	24	1.6	11.2	2.2	....	0	.....	6.3	.....	Decreased appetite.
3rd	24	1.3	.....	1.6	0.9	0.3	.....	5.0	178	Decreased appetite.
4th	22	1.3	12.0	1.7	0.8	0	47.3	3.8	.....	Decreased appetite.
5th	20	1.2	9.6	1.6	0.7	0	45.0	4.1	.....	Decreased appetite.
6th	25	1.1	.....	1.4	1.0	0	.....	....	.....	Decreased appetite.
7th	21	1.0	8.6	1.3	0.7	0	38.0	4.2	448	Decreased appetite.
Dose of 100 Gm. of sulfanilamide plus 1 Gm. of Paba given										
8th	....	....	.....	1.6	0.4	....	.....	1.6	.....	Edema under abdomen; muscle rigidity.
Dose of 50 Gm. of sulfanilamide plus 1/2 Gm. of Paba given daily										
9th	23	1.1	9.7	1.4	3.2	0	32.9	3.2	210	Edema under abdomen; muscle rigidity.
10th	21	1.3	9.7	2.0	0.3	0	46.0	3.9	.....	Edema still present.
11th	21	1.4	9.4	2.8	0.9	0	41.3	4.2	.....	Less edema.
12th	21	1.4	9.5	2.8	0.3	0	44.4	....	.....	Recovered.
No treatment given										
13th	24	2.3	11.5	3.1	0.6	0	48.0	5.1	.....	Recovered.
14th	....	2.8	10.9	4.3	0	0	.....	3.0	.....	Recovered.
15th	31	2.2	.....	3.6	0	0	.....	3.0	.....	Recovered.

	Significance of differences (mean values)	
	Inorganic phosphorus whole blood	Inorganic phosphorus plasma
Between pretreatment and treatment with sulfanilamide	$t = 7.934$†	$t = 5.935$†
Between treatment with sulfanilamide and treatment with sulfanilamide plus Paba	.....	$t = 2.384$*

* Probability of equal chance effect < 0.05; † probability of equal chance effect < 0.05 > 0.01.

toxic signs developing. After that time, 200 Gm. was given daily for four days, when slight signs of intoxication appeared in the form of excitement and a slightly unsteady walk; therefore, for four days, 2 Gm. of Paba was added to 200 Gm. of sulfanilamide. On the thirty-fifth day, the horse developed signs of myocarditis, which on necropsy, when later destroyed, was diagnosed as a hyaline degeneration of the heart muscle.

Inorganic phosphorus in the whole blood, plasma, and red blood cells did not alter materially during the treatment, but ester phosphorus in the whole blood and red blood cells, which decreased during the sulfanilamide treatment, increased again when Paba was added. During the administration of sulfanilamide there was a decrease of the phosphatase values. Elimination of phosphorus in the urine initially increased greatly during the administration of sulfanilamide but decreased again.

Case 7 (Table 8).—This 590-kg. (1,300-lb.) horse was given 100 Gm. of sulfanilamide twice daily for two days and, on the third day, signs of increased irritability appeared. The dose was decreased to 100 Gm. daily and the signs disappeared. This dose level was continued for 15 days without any signs of intoxication. Then the dose was increased again to 200 Gm. daily for five days. As early as the first day of this increased dose, the horse showed depression, muscular stiffness, and ataxia. The next day the horse became worse, fell, and had to be helped to his feet. The following day, he spontaneously improved. Two days later, severe depression developed and, on that day, the dose was decreased to 100 Gm. of sulfanilamide combined with 1 Gm. of Paba. The next day, this dose was doubled but the toxic symptoms disappeared and did not recur during the following five days of the experiment.

In this horse, inorganic phosphorous fractions in the whole blood and plasma increased on the twenty-second day, when the symptoms became severe. The ester phosphorus in the whole blood and red blood cells showed a primary decrease and a secondary increase during the administration of sulfanilamide. Phosphatase values decreased markedly during administrations of sulfanilamide. There was a statistically significant increase in urinary phosphorus during the administration of sulfanilamide but a decrease again when Paba was added.

TABLE 7 (Case 6)—Effect of Sulfanilamide (100 Gm. for 27 Days Plus 200 Gm. for 4 More Days) on a 582-kg. (1,283-lb.) Horse

Day of experiment	Hematocrit	Whole blood		Plasma		Red blood cells		Alkaline phosphatase (units/100 cc.)	Phosphorus elimination in urine (mg./day)	Clinical symptoms
		Inorganic phosphorus (mg./100 cc.)	Ester phosphorus (mg./100 cc.)	Inorganic phosphorus (mg./100 cc.)	Ester phosphorus (mg./100 cc.)	Inorganic phosphorus (mg./100 cc.)	Ester phosphorus (mg./100 cc.)			
Normal values without treatment										
1st	30	2.2	14.2	2.9	0.4	0.4	48.2	5.6	70	.....
2nd	30	2.4	15.2	2.7	0.3	0.2	47.1	6.2	90	.....
Dose of 100 Gm. of sulfanilamide given daily										
1st	....	....	.....	....	....	....	.....	....	1,032	.....
2nd	....	....	.....	....	....	....	.....	....	1,958	.....
3rd	....	....	.....	....	....	....	.....	....	100	.....
Dose of 200 Gm. of sulfanilamide given daily										
28th	30	2.0	14.5	2.8	0	0.2	48.3	2.1	106	.....
31st	28	2.5	10.7	2.9	0	1.6	38.1	3.7	92	Excitement; slightly unsteady walk.
Dose of 200 Gm. of sulfanilamide plus 2 Gm. of Paba given daily										
32nd	27	2.2	11.8	2.8	0	0.6	43.8	3.4	.....	.....
33rd	26	2.3	13.3	3.0	0	0.2	51.3	3.9	234	.....
35th	26	2.6	.....	3.2	0	0.8	.....	3.0	.....	Severe heart failure; unable to walk.
36th	28	2.5	14.5	2.9	0	1.4	51.8	3.3	340	Severe heart failure; unable to walk.
No treatment given										
37th	28	2.6	14.4	3.5	0	1.8	51.4	3.1	.....	Severe heart failure; unable to walk.
40th	23	1.9	10.6	2.0	0	1.4	46.3	3.3	.....	Severe heart failure; unable to walk.
43rd	26	1.8	8.8	2.2	0	0.7	33.8	3.4	.....	Killed.

Conclusions

Sulfanilamide given to horses as a single dose of 0.25 Gm. per kilogram (2.0 gr./lb.) of body weight, produced a highly significant decrease in inorganic phosphorus in the whole blood and plasma. If 0.0025 Gm. of Paba per kilogram of body weight was added to this dose, of sulfanilamide, the drugs had no effect on the inorganic phosphorus in the whole blood. Thus, Paba appeared to counteract the effect of sulfanilamide on the inorganic phosphorus of the whole blood.

When repeated doses of sulfanilamide were given to 7 horses, inorganic phosphorus in the whole blood and plasma usually decreased. In two of the experiments, the decrease was highly significant (cases 1 and 5). After Paba was added, there was a highly significant increase in inorganic phosphorus in plasma in case 1 and a significant increase in case 5. The administration of sulfanilamide usually increased the elimination of phosphorus in urine. The average increase was highly significant, but was greatest in cases 1 and 7. Considered individually, the cases show a temporary effect on most of the phosphorus fractions, the level of phosphatase, and the amount of phosphorus eliminated in the urine. In experiments 1, 4, and 6, for example, urinary phosphorus showed a very marked increase under the influence of sulfanilamide. In experiments 4 and 6, the phosphorus values in the urine spontaneously returned to normal. The same tendency had earlier been observed in experiments with rats when the lethal effect of sulfanilamide was tested.

Sulfanilamide caused the death of 2 ani-

TABLE 8 (Case 7)—Effect of Sulfanilamide (100 or 200 Gm. Daily) on a 590-kg. (1,300-lb.) Horse

Day of experiment	Hematocrit	Whole blood: Inorganic phosphorus (mg./100 cc.)	Whole blood: Ester phosphorus (mg./100 cc.)	Plasma: Inorganic phosphorus (mg./100 cc.)	Plasma: Ester phosphorus (mg./100 cc.)	Red blood cells: Inorganic phosphorus (mg./100 cc.)	Red blood cells: Ester phosphorus (mg./100 cc.)	Alkaline phosphatase (units/100 cc.)	Phosphorus elimination in urine (mg./day)	Clinical symptoms
Normal values without treatment										
1st	28	2.9	21.2	3.7	....	1.3	.....	6.8	67	.....
2nd	33	2.0	22.5	3.4	0	1.3	56.3	7.9	55	.....
3rd	34	2.8	24.1	3.7	....	1.1	54.2	6.4	76	.....
4th	....	2.8	.....	....	....	....	.....	7.6	.....	.....
Dose of 100 Gm. of sulfanilamide given twice daily										
1st	41	2.4	20.8	3.1	0	1.4	50.8	6.6	.....	.....
2nd	....	....	.....	....	....	....	.....	....	.....	.....
Dose of 100 Gm. of sulfanilamide given daily										
3rd	41	3.0	20.3	4.1	0	1.5	56.2	5.9	.....	Irritability.
4th	39	2.9	20.6	4.0	0.1	1.5	60.5	7.7	.....	.....
5th	35	2.8	15.8	3.7	0.1	1.2	44.8	6.9	.....	.....
8th	36	2.6	14.4	2.9	0	1.9	40.0	....	.....	.....
9th	36	2.8	14.2	3.1	0	2.3	39.4	7.2	356	.....
15th	36	2.8	19.4	3.3	0	2.0	53.9	4.6	260	.....
16th	39	2.7	20.0	3.3	0	1.7	51.5	3.9	.....	.....
Dose of 100 Gm. of sulfanilamide given twice daily										
17th	41	2.2	21.4	4.1	0	0	52.3	3.6	210	Depression; muscular stiffness; ataxia.
18th	33	2.8	18.0	3.0	0	2.5	54.5	3.8	189	Fell, had to be helped to its feet
20th	39	2.3	19.6	2.8	0	1.5	50.3	4.9	.....	Improvement.
21st	39	3.1	21.9	3.3	0	2.8	56.2	3.7	750	.....
Dose of 100 Gm. of sulfanilamide plus 1 Gm. of Paba given										
22nd	38	3.7	21.3	4.6	0	2.3	55.8	2.9	.....	Severely depressed.
Dose of 100 Gm. of sulfanilamide plus 1 Gm. of Paba given twice daily										
23rd	41	1.6	20.1	2.2	0.8	0.7	48.0	4.3	102	.....
24th	42	2.9	19.1	3.2	0.3	2.6	45.0	4.2	.....	.....
25th	40	2.7	17.8	3.6	0	1.3	44.4	3.0	.....	.....
26th	41	2.8	.....	3.7	0	1.6	.....	3.5	.....	.....
27th	34	2.5	18.8	3.2	0.1	1.1	55.0	....	.....	.....

	Significance of differences (mean values): Urine phosphorus
Between pretreatment and treatment with sulfanilamide	$t = 4.950$*

* Probability of equal chance effect < 0.05 > 0.01.

mals and severe heart failure in a third. The most common clinical signs of the toxic effect of sulfanilamide were depression, muscular stiffness, and allergic signs, such as urticaria and edema. Depression appeared in cases 1, 2, 3, 4, 7, and 8. Any uniform effect on the phosphorus metabolism in relation to this sign was not seen, except that phosphorus elimination in the urine was increased. Muscular stiffness appeared in cases 4, 5, and 7. It was impossible to correlate phosphorus metabolism with the time of appearance of the signs. Urticaria and edema, which appeared in cases 2, 3, 4, and 5, had no observable effect on phosphorus metabolism. Therefore, it can be concluded that the effect on the phosphorus metabolism is not the main effect of sulfanilamide and is not of primary importance in the appearance of clinical signs of intoxication.

The effect of sulfanilamide on the phosphorous fractions was not accompanied by any damage to the kidneys. Careful histological examinations of the 3 dead horses showed no sign of kidney damage, nor was there even any clinical sign of disturbed kidney function in the other horses. Urine was carefully checked, but no albumin was observed, nor was there any change in daily output.

Selye[8] has shown that the nonspecific responses of the body to harmful influences (stress) follow a definite pattern which he calls the "general adaptation syndrome." Various agents (stressors) can call forth these reactions which include both pathological and biochemical changes. It is not unreasonable to look upon sulfanilamide as a stressor which causes, among other things, a disturbance in phosphorus metabolism.

According to Selye,[8] the general adaptation syndrome is the sum of all nonspecific reactions of the body which ensue upon long-continued exposure to stress. The effect of sulfanilamide on phosphorus metabolism is, in our opinion, one of the systemic reactions of which the general adaptation syndrome is composed.

The toxic signs appeared at different times after the administration of sulfanilamide was begun. This great variation in individual reactions is the most important discovery of these experiments. It was demonstrated experimentally that one can not predict when toxic signs will appear in connection with sulfanilamide therapy. The toxic effect of sulfanilamide is of economic importance, since 2 horses died and severe cardiac damage resulted in a third.

SUMMARY

Sulfanilamide, given as a single dose to horses, decreased inorganic phosphorus in the whole blood and plasma. When para-aminobenzoic acid (Paba) and sulfanilamide were given together, there was no such effect.

When repeated doses of sulfanilamide were given to horses in seven experiments, severe toxic signs appeared in some animals. Two horses died, 1 with colic and 1 with multiple embolisms. One horse had to be destroyed because of myocarditis but the other 4 animals recovered either when Paba was added or when the administration of sulfanilamide was stopped. The most common effect of repeated doses of sulfanilamide on the phosphorus metabolism was an increase in inorganic phosphorus in the whole blood and plasma and phosphorus elimination in the urine.

To understand the contrary effect of single and repeated doses of sulfanilamide on phosphorous fractions in the blood, it is necessary to remember Selye's hypothesis that these effects represent different stages in the same process—the general adaptation syndrome.

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Methods of Spinal Fusion and Vertebral Immobilization in the Dog

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MANY veterinarians from time to time have seen the need for the development of surgical techniques to produce spinal fusions or vertebral immobilization or both. Vertebral fractures in animals, in the great majority of cases, have terminated in death or permanent paralysis. Intervertebral disc surgery in the dog, as described by veterinary surgeons,[1-4] has been unaccompanied by conjunctive stabilization or fusions of the spine. With these thoughts in mind, the author has endeavored to devise in the dog techniques that will produce vertebral immobilization and spinal fusion. They are described in this paper.

LITERATURE

Armstrong[5] has outlined the basic conditions of bone grafting in man as follows:

1) There should be close and stable contact between large areas of raw bone on the graft and host.
2) Fixation of the graft and fracture must be mechanically stable.
3) Before grafting, the fractured surfaces should be cleared of fibrous tissue and sclerotic bone.
4) Normal apposition and alignment of the fragments of the host bone should be restored.
5) The architecture of the reconstructed bone as a whole should as far as possible approximate to normal at the conclusion of operation.

There are various opinions as to the most effective type of bone to use and how it should be placed or fixed. Campbell[6] states that osteogenic cells and osteocytes survive only in fresh autogenous bone grafts. Autogenous bone grafts are also better tolerated in tissue than preserved or fresh homogenous grafts. Wilson and Barth,[7,8] on the other hand, have suggested that healing of bone-bank grafts and fresh autogenous grafts largely takes place by a process of invasion, absorption, and replacement. Both of these types are indistinguishable several months after implantation; however, fresh autogenous grafts become incorporated more rapidly. Gallie and Robertson[9] state that such osteoblasts that stay alive do so from their access to a surrounding supply of blood and lymph. They begin to proliferate and make new living bone along the framework of bone that has died for lack of fresh blood and lymph.

If growth is to take place, the bone should be so cut, prepared, and placed as to take greatest advantage of the revascularization process. The graft[5] should be in contact with a vascular area, and the deeper layers of the cortex and medullary cavity are more vascular than the superficial cortex. The graft will be more easily vascularized if it contains, at least in part, soft cancellous endosteal bone which is in immediate contact with the host bone. Finally, the larger the areas of graft and host brought in contact, the more rapid will be the revascularization of the graft and its subsequent replacement by living bone. Since osteoblasts are most prevalent under periosteum and endosteum, osteogenesis will take place much more rapidly if one or both of these structures can be included in the graft.[10]

In general, it would seem from the literature that fresh autogenous grafts give a "faster take" than homogenous or bank grafts but that the end result would be much the same. A graft should be prepared to take advantage of the most abundant supply of osteoblasts as well as of blood for nutrition.

The types of grafts used in spinal work are briefly as follows:

1) Intervertebral bone graft[11] is a graft inserted between the bodies of the vertebra in the disc space (fig. 1).

2) Clothespin graft[12] is an "H"-shaped graft wedged between the spinous processes with the spines so "notched" as to keep the graft fixed (fig. 2.)

3) Hibb's method[13] was one of the first successful methods and consists of splitting the spinous processes into small fragments, chipping the outer surfaces of the laminal arches, removing the cartilage from the articular facets, and placing the bone chips as bridges across the denuded bone of the vertebral arches. Additional bone chips from the ilium or tibia can be used to reinforce the area.

4) Albee's method[14] is a "wedge" or an "inclusion" graft which consists of placing a rigid graft between the split spinous processes and securing it by means of wire or other suture material (fig. 3).

5) Onlay graft[5] is used frequently on long bones

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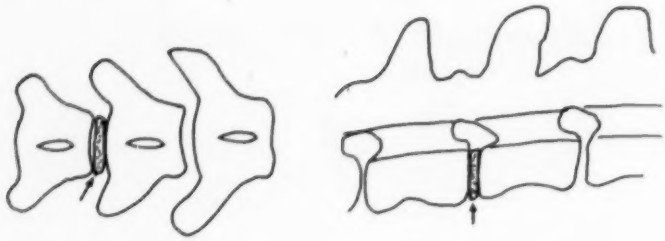

Fig. 1—In the intervertebral bone graft, the disc is completely removed and a comparable flat, disc-shaped, section of bone (arrows) is forced in the intervertebral space.

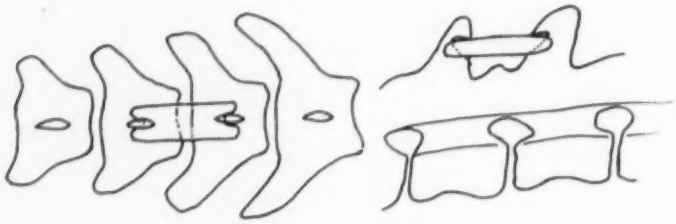

Fig. 2—The clothespin or H graft is a properly shaped piece of cortical bone forcibly placed between the notched spinous processes.

and consists of placing a rigid bone graft on the prepared surface of the host bone and securing it tightly with screws or similar items (fig. 4).

PROCEDURE

The investigation of spinal fusions and immobilization methods in the dog was accomplished in a two-year period, 1953 and 1954. During 1953, the single and double onlay and H, or clothespin, bone graft methods were employed in 4 dogs. The postsurgical observation period was three months. One graft established itself well while two others probably would have if they had been given more time. It was determined that the last rib was the most accessible bone for grafting and was more suitable as far as vascularity was concerned than a section of tibia (dogs 1, 2, 3, 4, table 1).

Spinal immobilization methods used in 1953 employed vitallium bone plates and Wilson laminectomy plates, both of which were applied to the spinous processes. Bone plates and screws placed in 2 dogs gave only fair stabilization but were well tolerated by the tissues (dogs 15, 16, table 1). Wilson laminectomy plates and bolts were applied to the dorsal lumbar spines of 2 dogs (17, 18, table 1). These plates gave much better support than the plates and screws and were also well tolerated by the tissues.

The follow-up study in 1954 was undertaken with the following objectives in mind:

1) To repeat the bone grafting procedure and allow a six-month postoperative period for observation and evaluation.

2) To evaluate the union of these grafts in cases of external fixation and in cases where no immobilization was provided.

3) To further evaluate spinal fracture and luxation methods in experimentally produced or similated fracture cases. The intervertebral bone graft was not used because of the very small intervertebral space in the dog. The so-called "clothespin" graft will be referred to as the "H" graft and Albee's method will be called the "wedge" graft. Hibb's method was not accomplished but "onlay" or "lay on" grafts were investigated.

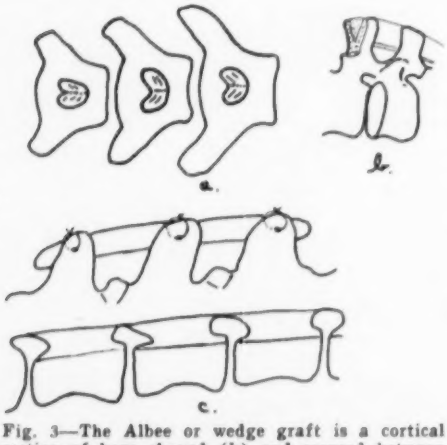

Fig. 3—The Albee or wedge graft is a cortical section of bone shaped (b) and secured between the split (a) spinous processes with wire or other suitable material (c).

1954 RESULTS

BONE GRAFTS

Generally, the experimental cases were treated as similarly as possible. The anterior lumbar vertebrae were used [the first lumbar (1L), second lumbar (2L),

TABLE 1—Bone Grafts Using Various Methods of Immobilization in the Dog

Dog	Procedure	Observation period	Spinograph findings	Necropsy findings	Histological findings	Conclusions
			BONE GRAFTS WITH NO EXTERNAL FIXATION			
1*	Tibial bone graft onlay, on each side of 3L-4L** dorsal spine fastened with nylon 00	4 mo.	.....	Good take, somewhat smaller, added little rigidity to spine	Some evidence of bone formation from fibrous connective tissue present	Satisfactory take
2*	Rib graft, single onlay fastened to 4L-5L spine by means of bone screws	2 mo.	Showed longitudinal splitting of graft on one end; retained position and size for 2 mo.	Dog lost	.....	Probably a take
3*	Double onlay rib graft to 2L-3L spines secured by means of bone screws	3½ mo.	Bone fragments retained size and density	Bone fragments came loose when screws were removed	Fibrous tissue; breakdown and formation of bone	Not a complete take, screws in grafts maintained some rigidity
4*	H rib graft forcibly placed between the 2-3L† dorsal spines (fig. 6)	3 mo.	Gradual absorption of graft	Graft was largely replaced with cartilage and fibrous-like tissue	Heavy fibrous tissue; some evidence of osseous development	Poor take; no extra support gained
5	A single onlay rib graft applied to 1L-2L dorsal spines with Kirschner wire	5½ mo.	Maintained density and position	Dog lost after 5½ months	None	Good take probable
6	An H rib graft was forcibly placed between 1-2L spines; 2 small onlay grafts were also placed on each side of dorsal spines*	3½ mo.	H maintained density and position; onlays not seen well	Dog lost after 3½ months	None	Take probably
7	A single onlay graft was secured to 1L and 2L with nonthreaded ⅛-in. dia. pins at junction of lateral processes and vertebral bodies	3 mo.	Posterior pin loosened and this end of graft not in place	Secured end showed a take; the loose end not in place	None	Partial take
8	A wedge type rib graft placed between 1-2L split dorsal spines and secured with nylon	6½ mo.	Original graft degenerated but secondary exostosis developed	Secondary exostosis gave excellent support and rigidity	Much fibrocartilage and new bone present; fragments of graft tissue being removed by osteoclasts	Good take; good support from secondary bone formation
			BONE GRAFTS WITH EXTERNAL FIXATION			
9	H rib graft placed between 1-2L spines; small double onlay grafts loosely placed on each side of dorsal spines; aire-cast, 33 days	6 mo. 3 days	Very slight degeneration of grafts	Grafts well established grossly; some rigidity to area	Graft bone transformed into connective tissue, cartilage, and new bone	Good take; some support
10	Wedge graft placed between split dorsal spines of 1L and 2L and secured with nylon; aire-cast, 36 days	6½ mo.	Gradual degeneration of original graft with a secondary bony proliferation	Graft taken well; exostosis gave good rigidity	Entire section contained mature bone; complete transformation	Good take; good support
11	A single onlay graft to the 1L-2L dorsal spines secured with Kirschner wire; aire-cast, 40 days	6¼ mo.	Graft maintained itself; good bone bridge between vertebrae	Graft well established; rigidity present	Some graft bone removed by osteoclasts; fibrocartilage and new bone around graft areas	Good take; fair rigidity
12	A double onlay graft placed on each side of 1L-2L spinous processes and secured with 0 stainless steel suture; aire-cast, 8 weeks	7 mo.	Gradual regression in size of graft; bone formation and fusion evident	Graft well established; some rigidity to spine	Some graft bone removed by osteoclasts; fibrocartilage and new bone around graft areas	Good take; some rigidity to spine

TABLE 1 (Continued)—Bone Grafts Using Various Methods of Immobilization in the Dog

Dog	Procedure	Observation period	Spinograph findings	Necropsy findings	Histological findings	Conclusions
			BONE GRAFTS WITH BONE PLATES AS SKELETAL FIXATION			
13	A single onlay rib graft to the denuded articular process area of 1L-2L vertebra; stainless steel wire used; 3-cm. bone plate and Wilson bolts	7 mo.	Bone plate obliterated study of graft	Plate gave good rigidity; bone graft soft and not well incorporated	Transplant largely reabsorbed; cartilage and new bone laid down; much inflammatory reaction in bone plate area	Poor take; plate gave good support
14	A single onlay rib graft secured to the bodies of 3L-4L by means of bone screws; No. 1 Wilson plate and bolts secured to 3L-4L dorsal spines for immobilization	6 mo.	Lost some density but considerable was left at 6 months	Fair take although graft shrunken in size; fair rigidity when screws in place; poor when removed	No original bone graft tissue; moderate proliferation of periosteum and slight new bone	Fair take; rigidity largely lost when screws and plates removed
			FRACTURE IMMOBILIZATION METHODS ON NORMAL DOGS			
15	2 in. bone plate screwed to 5L-6L spines	3½ mo.	Normal	Screws loose; little immobilization	None	Not satisfactory immobilization
16	2 in. bone plate secured to ventral surface of 4L, 5L, 6L, through a laparotomy incision by means of bone screws	2 mo.	Screws protruded slightly into spinal canal	Animal lost	None	Difficult, but satisfactory method; not practical
17	Wilson No. 1 laminectomy plate secured to 2L-3L dorsal spines by means of bolts	2½ mo.	Normal	Plate well placed; no reaction; fibrous tissue around plate	None	Good immobilization
18	Wilson No. 2 plate placed on 1L, 2L, 3L dorsal spines by means of bolts	2 mo.	Normal	Fibrous tissue and bone proliferated around plate and bolts	Cartilage tissue around plate	Good immobilization
			FRACTURE IMMOBILIZATION METHODS, SIMULATED FRACTURES, PLATES ON SPINE			
19	Disarticulated 2L-3L; Wilson No. 1 plate and bolts applied to 1L, 2L, 3L	3 mo.	Good reduction; satisfactory immobilization	Bolts and plates secure; cord normal, grossly	Slight microscopic changes in dorsal ventral white substance	Good
20	Disarticulated at 1L-2L; small bone plate placed on 1L-2L dorsal spines secured with screws	1 day	1 screw and part of dorsal spine broken off; spinal cord	1 screw and part of dorsal spine broken off; spinal cord severed	Not done	Poor
21	Disarticulated at 2L-3L; 5 cm. bone plate placed on 2L-3L lateral processes by means of screws	5 days	Gross displacement of vertebrae cord pressure present	Anterior screw loose; allowed vertebrae to displace itself dorsally	Not done	Poor results
22	Disarticulated at 2L-3L; 2 Wilson No. 2 plates secured to 1L, 2L, 3L dorsal spines with bolts	5 mo.	3 weeks after surgery bolt and part of 3L dorsal spine broke loose; in a few weeks dog compensated clinically for pressure and could walk	Much bone callus and exactions existed at area; 2L was displaced dorsally approx. 5mm.	White substance in ventral and lateral columns contains large scattered vacuoles, fragment of tissue debris, and occasional phagocytes	Good until dorsal spine broke due to extreme exercise; a large active dog
23	Disarticulation at 2L-3L; 2 bone plates (10 and 6 cm. long) on each side of 2L-4L dorsal spines, secured with bolts	4 mo.	Normal	Much fibrous and bony tissue around plates and bolts; floor of canal smooth, no cord sections	Not done	Good

TABLE 1 (Continued)—Bone Grafts Using Various Methods of Immobilization in the Dog

Dog	Procedure	Observation period	Spinograph findings	Necropsy findings	Histological findings	Conclusions
			Fractures Immobilized by Other Means			
24	Disarticulation at 2L-3L; hole drilled in each dorsal spine and double strand of 00 stainless steel wire placed in the spines	3 mo.	Good apposition and healing	Dog disappeared at time of proposed necropsy	Not done	Good
25	Disarticulated at 2L-3L; surgical wound closed and plaster of paris cast applied for 4 wk.; aire-cast applied later	3 mo.	Good apposition and gradual healing	Slight constriction of cord at necropsy; scattered demyelination in dorsal and ventral white substance in this area	Not done	Good
26	Disarticulated at 3L-4L; bone plate (5 cm.) placed on ventral surface of 3L-4L vertebrae through laparotomy incision	None	None	Dog destroyed because of damage to vena cava in placing the	Not done	Not successful

* These dogs were observed during 1953, all others were observed during 1954; ** 3L-4L—on third and fourth lumbar vertebrae; † 2-3L—between second and third lumbar vertebrae.

etc.]. The bone grafts were all secured from the last rib. Spinographs were taken every two weeks for two months and once every month thereafter. The cases are described as follows:

Bone Grafts with External Fixation.—Experimental dog 9 was a 22-lb. male of mixed breeding, 3 to 5 years old. An H graft was placed between the 1L and 2L vertebral spinous processes. In addition, two slender sections of bone were placed as onlay grafts on each side of the spines. The grafts were secured from the last rib and the periosteum was peeled back from contact surfaces. A plastic cast * was applied to the trunk extending from the shoulder to the preputial region. The cast was left on, except for periodic dressing of the incision, for 33 days.

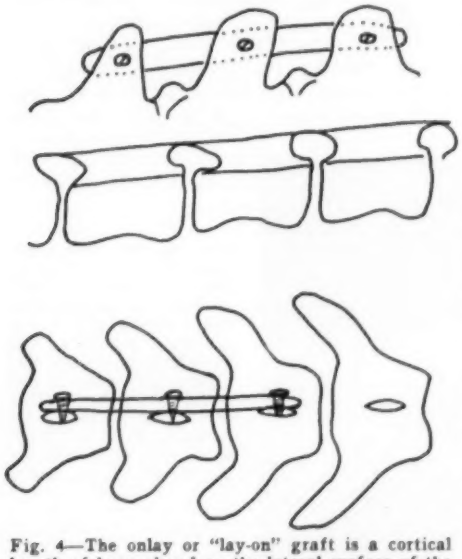

Fig. 4—The onlay or "lay-on" graft is a cortical length of bone placed on the lateral surface of the spinous processes and secured with screws or wire material.

The recovery progressed and periodic spinographs were taken at 2-, 4-, 6-, and 8-week intervals and at monthly intervals thereafter. All radiographs demonstrated a persistence of the grafts with but slight dissolution (fig. 5). The animal was destroyed after six months. The grafts were in place with one end well incorporated, but the other end was not firm. The graft had given some rigidity to the area. On histological examination, the transplant bone had been transformed into intermediary forms, such as connective tissue, cartilage and, finally, bone.

Experimental dog 10 was a 13-lb. Setter bitch, about 4 months old. A wedge-type graft was placed between the split spinous processes of 1L-2L vertebrae. The graft was a rib section with marrow bone exposed to one side and cortical bone with the periosteum peeled back on the opposite side. Small holes were drilled in each spine through the graft as placed and secured

* Aire-Cast, Tower Co., Inc., Geneva, Ill.

Fig. 5—This spinograph was taken six months after surgery on experimental dog 9. The H graft was giving some rigidity but one end was not firm.

with No. 00 monofilament nylon suture. A plastic cast was placed as described for dog 9 and was left on for 36 days. The dog, however, would remove the cast and it was replaced repeatedly.

Progress was satisfactory and spinographs were taken at regular intervals as described for dog 9. They showed a progressive loss of original graft contour which was superseded by a large proliferation of bony tissues in the area. The 1L-2L vertebrae were nearly completely fused by a massive section of bone at the end of the observation period (fig. 7). The animal was destroyed after six and a half months. The section of spine 1L-2L was rigidly immobile and was partly immobile at 13T-1L. The graft apparently had "taken" completely. In the histological sections, no differentiation of transplant bone could be distinguished. The entire section looked like mature parent bone. The transplant had completely established itself.

Experimental dog 11 was a 20-lb. female of mixed breeding, about 1 to 2 years old. An onlay type rib graft was applied to the side of the spinous processes of 1L-2L vertebrae and kept in place by clinching a Kirschner wire section through the graft and process. The periosteum was peeled off each contact surface. A plastic cast was applied as previously described and was removed after 40 days. Much trouble was experienced in keeping it on effectively. Periodic spinographs showed that the graft was maintaing itself. It finally formed a good bony bridge between the involved vertebrae.

The animal was destroyed after six and a quarter months and, grossly, the graft

Fig. 6—This spinograph demonstrates an H graft one week after surgery in 1953 on experimental dog 4. After three months, the graft was largely replaced by cartilaginous and fibrous tissue which was adding no support to the spinal column. A longer postoperative period possibly would have shown better fusion.

Fig. 7—This spinograph was taken six and a half months after transplantation of the wedge graft in experimental dog 10. A massive and solid fusion had occurred between the spinous processes in which the wedge graft had been placed.

looked secure and well established. The Kirschner wire segments were securely in place and were causing no reaction. The graft was adding rigidity to the spinous area (fig. 8). In the histological sections, fragments of bone graft tissue were being removed by osteoclasts. Considerable amounts of fibrocartilage and new bone had developed around the remnants of the graft.

Experimental dog 13 was a 10-lb. male of mixed breeding, 3 months old. A rib section was utilized as an onlay graft on the articular processes of 1L-2L vertebrae. The graft was secured with No. 00 stainless steel wire sutures. A small bone plate (3 cm. long) with Wilson laminectomy bolts was placed on the respective spinous processes as a means of immobilization (1L-2L). The periosteum of the graft was peeled off the contact surfaces. Periodic spinographs were not diagnostic since the bone plate largely obliterated the graft; however, there seemed to be a gradual filling in of bone below the plate in the region of the graft. Progress was satisfactory.

The dog was destroyed after seven months. The plate and bolts were firmly in place and were affording excellent rigidity. However, the bone graft seemed soft and was not well incorporated into the parent bone. Histologically, the transplant had largely been reabsorbed and cartilage and new bone was being laid down. The area adjacent to where bone plates had been applied showed a marked inflammatory reaction.

Experimental dog 14 was a 40-lb. male of mixed breeding, from 2 to 3 years old. An onlay graft was secured to the bodies of the 3L and 4L vertebrae with bone screws. These screws were a half inch long and were placed into the graft and the vertebrae after a hole (1/8-in. dia.) had been drilled through these structures. A Wilson No. 1 laminectomy plate was secured to the 3L and 4L spinous processes by laminectomy bolts. This plate

Fig. 8—This spinograph was taken six and a quarter months after a section of rib of experimental dog 9 had been placed as a single onlay graft on the lateral side of the dorsal spines and secured in place with heavy wire. Good fusion and rigidity was established.

was to serve as a means of immobilization. The periosteum was scraped back from contact surfaces. Periodic spinographs showed that the bone graft gradually lost much of its density. A ventrodorsal view revealed a substantial amount of bone left in the graft. The clinical progress was satisfactory.

The dog was destroyed after six months. On gross appearance, the graft had shrunken but had incorporated itself fairly well. The union of the graft to the parent bone was not entirely firm when the screws were removed. The good rigidity present with the plate and the graft screws in place was largely lost when the plate and screws were removed. The microscopic sections revealed no original bone graft tissue but moderate proliferation of periosteum with slight bone formation.

Experimental dog 12 was an 8-lb. female of mixed breeding, about 2 to 3 months old. A rib section was cut into two similar sections and one was placed on each side of the spinous process of 1L and 2L (double onlay graft). The periosteum of contact surfaces was peeled back and the grafts were secured by No. 0 stainless steel wire sutures. Aire-cast was applied to the thoracolumbar trunk. The progress was satisfactory; however, the cast, which had to be replaced frequently, was observed to give little immobilization. It was removed after eight weeks. Periodic spinographs revealed a gradual regression in size of the graft but bone formation and fusion were evident.

The dog was in good condition when destroyed after seven months. The grafts were well established but added only slight rigidity to the spine since they were not particularly heavy or large. Histologically, some fragments of bone graft tissue were being removed by osteoclasts. Considerable amounts of fibrocartilage and new bone had developed around some remnants of the graft.

Bone Grafts Without External Fixation.—Experimental dog 5 was a 12-lb. male of mixed breeding, 2 to 3 months old. A rib onlay graft was placed on the 1L and 2L spinous processes after the periosteum was peeled back from contact surfaces. Kirschner wire (0.045 in. dia.) was drilled through the spines and the graft; it was then cut and clinched tightly by means of heavy forceps. No cast was applied.

The progress was satisfactory. Periodic spinographs showed the graft in place and maintaining its density. Spinographs taken at five and one-half months revealed a good "take." However, the dog was missing from its exercise run on the day it was to be destroyed.

Experimental dog 6 was a 25-lb. male of mixed breeding, 2 to 3 years old. A rib section in the form of an H graft was placed between the 1L and 2L spinous processes in a horizontal position. The dorsal spines were notched so that this graft would maintain its position. A small onlay graft was placed on each side of the spines in addition to the H graft. These onlays were not secured and a cast was not applied. The progress was satisfactory. Spinographs showed the H graft to be well in place, becoming well established, and maintaining its density. The onlay grafts were not clearly seen. The graft was lost after three and one-half months of observation.

Experimental dog 7 was a 43-lb. male of mixed breeding, 7 to 8 years old. A rib section onlay graft was applied to the 1L and 2L vertebrae where the lateral processes join the bodies. The graft was secured by nonthreaded intramedullary pins (⅛ in. dia.). These pins were drilled through the graft into the bodies of the vertebrae and the excess pin was removed with a pin cutter. No cast was applied. After two weeks, a spinograph showed that the posterior pin had loosened and migrated ventrally. Spinographs over a period of three months did not reveal a good graft. The dog was accidentally destroyed in another experiment.

Necropsy showed a good take of the graft at the point of contact of the remaining pin. The end of the graft where the pin was loose was not in place and was disintegrating. A histological study was not made.

Experimental dog 8 was a 20-lb. female of mixed breeding, 3 years old. The rib section was placed between split sections of the 1L-2L spinous processes in a wedge graft style. The cortical side of the graft was stripped of its periosteum. The graft was secured in place with No. 00 monafilment nylon suture. No cast was applied. The progress was satisfactory. Periodic

spinographs showed the original graft to be disintegrating; however, a secondary exostosis was developing in its place.

The dog was destroyed after six and one-half months. The graft seemed incorporated but the major support seemed to be from the exostosis in the area. The area was quite rigid and stable. Histologically, fragments of graft tissue were being removed by osteoclasts while considerable amounts of fibrocartilage and new bone were developing around the areas of bone graft.

SPINAL FRACTURE IMMOBILIZATION

The production of spinal fractures was a problem of deep concern in this investigation. It was decided that the most standard, humane, and efficient method was to surgically remove the entire bony support of the spinal cord. Here the dorsal and lateral laminae of the bony canal were removed with mastoid rongeur forceps and the intervertebral disc space entirely disarticulated. The spinal cord and its coverings were the only structures holding the spinal column together.

Fractures Immobilized with Bone Plates Placed on the Spinal Column. — Experimental dog 19 was a 10-lb. female of mixed breeding, 6 to 8 weeks old. The spinal column was disarticulated at the 2L-3L level. A small bone plate (5 cm. long) and laminectomy bolts were applied to 1L, 2L, and 3L spinous processes. The progress was satisfactory and the dog was walking the day after surgery. Periodic spinographs revealed good reduction alignment of the fragments and satisfactory immobilization throughout the observation period.

After three months, the dog was destroyed. The bolts were securely in place and were difficult to remove. Grossly, the cord looked normal. Histological sections revealed minimal degeneration of the dorsal and ventral white substance as indicated by occasional round single vacuolar spaces.

Experimental dog 20 was a 15-lb. female of mixed breeding, about 2 years old. The 1L-2L space was disarticulated and a small bone plate (5 cm.) was placed on the 1L and 2L spinous processes and was secured with screws. The day after surgery, there appeared to be a marked spinal displacement and a spinograph revealed that the cord had been severed by displacement of the plate. After euthanasia, necropsy revealed that one screw and part of a spinous process had broken off; the other screw was loose. No histological study was made.

Experimental dog 21 was a 15-lb. female Terrier, 1 year old. The 2L-3L space was disarticulated and a small (5 cm.) vitallium bone plate was placed on the 2L and 3L lateral processes by means of screws.

Progress was not satisfactory, and five days after surgery the dog was destroyed because of spinal pain and paraplegia. The spinograph showed a gross displacement of the vertebrae with resulting pressure on the cord. Necropsy revealed that both screws were still in place; however, the anterior one in 2L was loose and allowed this vertebra to become displaced dorsally. A histological study was not made.

Experimental dog 22 was a 30-lb. female, 2 to 3 years old. The 2L-3L space was disarticulated and two large Wilson laminectomy plates were secured with bolts to the 1L, 2L, and 3L spinous processes. The bolt and part of the 3L spine became loosened approximately three weeks after surgery. This resulted in upward displacement of approximately 5 mm. However, in a few weeks the dog compensated for this pressure and at the time of necropsy was quite normal, clinically.

After five months the dog was destroyed. Much bony callus and exostosis were present around the break. A section of the cord was taken at the 2L-3L intervertebral space. Histologically, the ventral and lateral columns showed areas of moderate nerve damage. The white substance contained large scattered vacuoles, fragments of tissue debris, and occasional phagocytes.

Experimental dog 23 was a 45-lb. female hound, 3 to 4 years old. The 2L-3L space was disarticulated and immobilized by using two bone plates bolted to 2L and 4L spinous processes; a bone plate 10 cm. long was on one side of the spine and a plate 6 cm. long on the other side. The progress was entirely satisfactory. Spinographs showed that a good reduction of fragments had occurred (fig. 9).

The dog was destroyed after four months. The plates were surrounded by bone and fibrous tissue and were most difficult to remove. The floor of the spinal

cord was smooth. A histological study was not made.

Fractures Immobilized by Other Means.—Experimental dog 24 was a 15-lb. tan female Spaniel, 3 years old. The 2L-3L intervertebral space was disarticulated. The immobilization procedure consisted of drilling a hole in each spinous process and placing a double strand of No. 00 stainless steel wire through the holes to hold the two vertebrae steady. The progress was satisfactory. No abnormal locomotion was seen. The periodic spinographs revealed good apposition and healing. The dog was observed clinically and radiographically for three months. At the time of proposed necropsy, the dog had disappeared.

Experimental dog 25 was a 25-lb. female of mixed breeding, about 6 months old. The 2L-3L space was disarticulated in the usual manner. The fixation consisted of a heavy plaster of paris cast applied to the thoracolumbar area. The dog was semiparalyzed at first but was walking at the end of ten days. She improved gradually until entirely normal. The plaster of paris cast did not harden and stayed semisoft. The cast was removed in a week and was replaced with an aire-cast. There is some doubt as to the exact efficiency of the immobilization casts since none fitted well. The cast was removed at the end of four weeks. The periodic spinographs showed good apposition and a gradual healing.

At necropsy, after three months, healing had taken place; however, there was some constriction of the cord at the fracture site. Histological examination revealed degenerative changes involving a moderate number of nerve fibers in both dorsal and ventral white substance. The white substance contained scattered elongated vacuolar spaces. Some of these were empty, others contained intact vacuoles, fragments of axones, or foamy phagocytic cells. Interpretations of the findings indicated moderate demyelination and, to a lesser degree, of axone degeneration in the white substance of the cord.

Experimental dog 26 was a 15-lb. female of mixed breeding, 2 to 3 years old. The 3L-4L space was disarticulated. An effort to immobilize the area was made by placing a 5-cm. bone plate on the ventral vertebral surfaces by means of a laparotomy incision. However in placing the screws in the vertebral bodies, the vena cava was injured and the dog was destroyed. This approach was considered as a possible but a dangerous one.

DISCUSSION

The bone grafts appeared to take better in those cases which had some immobilization. However, placing casts on dogs is extremely difficult and many of these dogs spent as much time out of the cast as in, since the cast would become loose and inadequate. Even when placed snugly on a female, it provided only partial immobilization.

Of the dogs with bone grafts without any external immobilization, 3 were lost from the exercise runs. However, spinographs of these dogs indicated that good unions were in progress. If we assume that the bone grafts in these lost dogs were successful, then five of eight were satis-

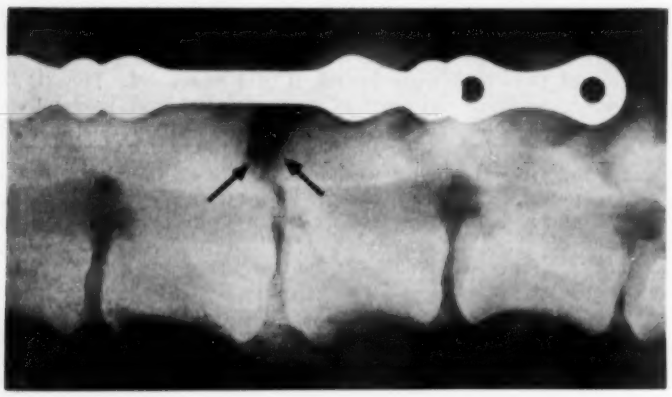

Fig. 9—In the fracture immobilization of experimental dog 23, the 2L-3L space (arrows) was disarticulated completely. Immobilization was effected by using a bone plate (6 and 10 cm. long) on each side of the spinous processes which were secured to the 2L and 4L spinous processes with bolts.

TABLE 2—Results of Bone Graft and Fracture Immobilization Methods

Method	Satisfactory (No.)	Fair (No.)	Poor (No.)
Bone graft without external immobilization	2 3*	2	1
Bone grafts with external immobilization	4	0	0
Bone grafts with bone plates on vertebrae	0	1	1
Bone plates on normal dogs			
Plates and screws to dorsal spinous processes	0	0	1
Plate and screws to ventral surface of vertebral bodies	1**	0	0
Wilson laminectomy plate and bolts	2	0	0
Simulated fractures			
Plates and bolts, dorsal spines	2	1	0
Plates and screws, dorsal spines	0	0	1
Plates and screws, lateral processes	0	0	1
Plates and screws, ventral surface of vertebral bodies	0	0	1
Wiring dorsal spines together	1	0	0
Plaster and aire-cast to body surface	1	0	0

* Probably satisfactory, no necropsy; ** difficult technique.

factory, two fair, and one poor. Since the results of those with immobilization seem better, some means of external fixation seems indicated.

In those dogs in which bone plates and bolts were placed on the adjacent spinous processes in addition to the graft, the results were not encouraging. In 2 dogs in this group, one graft resulted in a fair take and one in a poor take. Again, the number involved is small but indications are that the complete immobilization gained by this method is not advantageous for the bone graft. It would seem as if some movement is necessary for a take. However, the support gained by use of a plate is immediate, whereas support from a fusion or graft does not take place until six to 12 months have elapsed (tables 2 and 3).

The onlay-type graft was the most frequently applied. In consulting table 3 and the context, it will be seen that in both animals, wedge grafts produced a secondary exostosis and bone formation that gave an excellent support and rigidity to the area. From a clinical standpoint, the wedge graft seems to give the most support to the spine. Whether this bone will continue to proliferate exuberantly over a longer period could not be determined in this observation. The other type grafts listed as satisfactory were "good" takes but the graft itself was usually not large enough to supply much support to the spinal column.

TABLE 3—Results with Different Types of Grafts

Method	With external cast	No cast
Onlay		
Satisfactory	1	3
Fair	1	2
Onlay grafts with plates bolted to the spinous processes		
Satisfactory	0	0
Fair	0	1
Poor	0	1
Wedge		
Satisfactory *	1	1
H		
Satisfactory	1	1
Poor	0	1

* Gave best support and rigidity because of secondary exostosis.

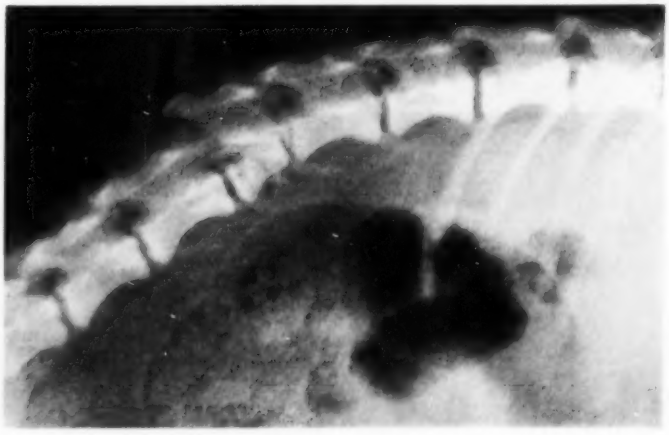

Fig. 10—Spinograph of a compression fracture of the 4L vertebra of a 2-year-old male dog (clinical case 1).

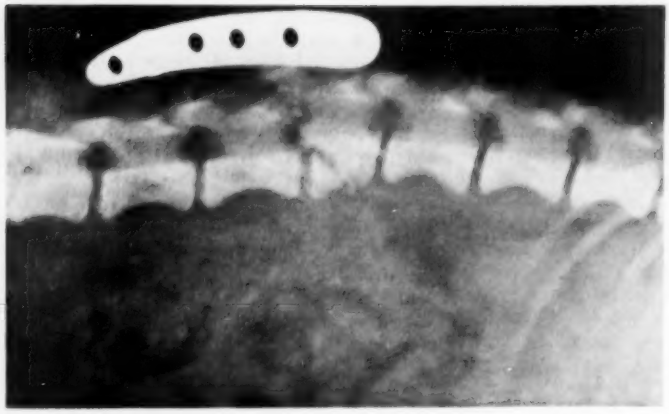

Fig. 11—Spinograph of clinical case 1 taken four weeks after fixation of the compression fracture with Wilson laminectomy plates and bolts (the bolts do not show on a lateral view). Notice the callus formation.

The fracture immobilization methods on simulated fractures demonstrated that screws and plates placed anywhere on the spinal column were not satisfactory. Plates and bolts well placed on the dorsal spines were satisfactory if the extreme stress applied to it did not break the dorsal spine. To avoid this stress, it might be well to use an external splint on large dogs, if possible. Wiring the dorsal spines together was successful in 1 male dog, and plaster and aire-cast was successful in a female; however, these were small dogs. According to the results of plates and bolts on three clinical cases, 2 of which were successful and 1 fatal (due to complications), this method would seem to be preferable. It might be advisable in some cases to combine more than one method, such as wiring dorsal spines together, plating the dorsal spines, and possible external splintage as well.

Clinical Cases with Spinal Fractures.—Case 1.—A 2-year-old male dog of mixed breeding was hit by a car and initially showed drooling, subconjunctival hemorrhage in the right eye, nystagmus of both eyes, severe abdominal pain, and cyanosis.

The animal was placed under observation, after a diagnosis of brain concussion, and was treated for shock. In 24 hours, it seemed improved but still had extreme pain in the abdomen. After eating a full meal, the dog would cry in pain for 15 to 30 minutes. He was able to stand, but moved his hindlegs reluctantly.

A normal contrast spinograph (fig. 10) revealed a fracture of the 4L vertebra with a dorsal displacement of the vertebra sufficient, it would seem, to cause a paralysis.

Treatment.—Since the dog was not totally paralyzed, a reduction was attempted. The results of previous experimental work indicated the application of a pair of Wilson laminectomy plates for spinal immobilization.

An incision, approximately 6 inches in

Fig. 12—Spinograph of the ventrodorsal view of the fixation of the compression fracture in clinical case 1 (fig. 10, 11).

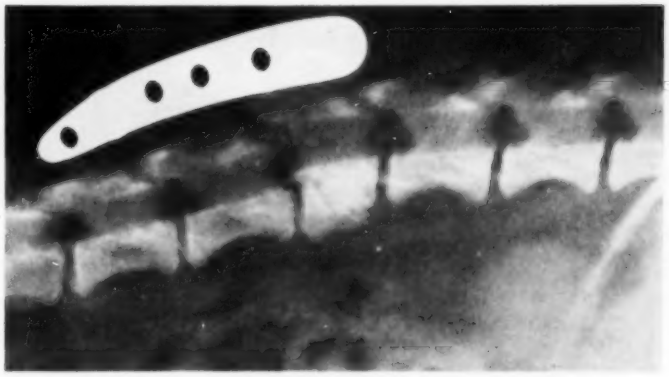

Fig. 13—Spinograph of clinical case 1 taken eight weeks after correction of the compression fracture. Notice the complete callus formation.

length, was made directly over the fourth lumbar spinous process. The musculature was removed from the 4L, 5L, and 6L spinous processes by blunt dissection. A No. 1 Wilson laminectomy plate was then laid on each side and secured with bolts placed in 6L and 4L spinous processes. During this manipulation, the dorsally displaced fragment was forced ventrally in an effort to remove pressure on the cord (fig. 11 to 13).

The animal chewed the incision open on the third day, but it was resutured and recovery was satisfactory. In three days, the dog was walking normally. The postoperative care included the administrations of mephenesin, thyroid, and calcium. Four weeks after surgery, the dog appeared entirely normal. A spinograph taken eight months later showed that the fractured vertebra was healed. An anteroventral bony bridge to the adjacent vertebra was being formed to further immobilize the section (fig. 14).

Case 2.—A male Boston Terrier, 3 years old, had shown complete posterior paraplegia for one week. The signs included urine and fecal retention, spastic paraplegia, exaggerated tendon reflexes, and extreme pain in the midthoracic spinal segment.

A spinograph revealed a compression fracture of the 6T vertebra, causing a compression myelitis.

Treatment consisted of placing a No. 1 Wilson laminectomy plate on the spinous processes with bolts in 5T and 8T (fig. 15). This plate immobilized the spinal segment and afforded a chance for healing. During the six months following surgery, the dog gradually improved until he could walk well but could not defecate and urinate entirely normally.

Case 3.—A bitch, 5 years old, hit by a car three days previously, showed a spastic paraplegia, urine and fecal retention, and severe pain in the region of the 12th thoracic vertebra.

The spinograph revealed a narrow intervertebral space at 11T-12T, a slight compression fracture of the 12T vertebra, and a separation of the articular proc-

Fig. 14—Spinograph of clinical case 1 taken eight months after correction of the compression fracture. Notice how complete healing has taken place with a marked ventral spur (spondylitis) occurring.

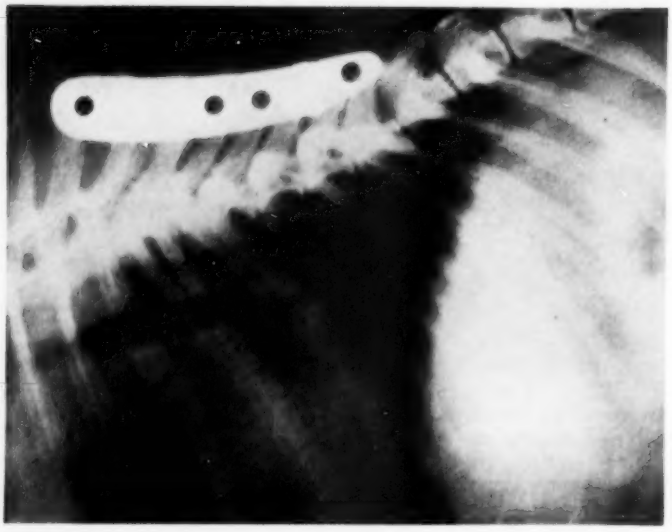

Fig. 15—Spinograph of clinical case 2 showing a compression fracture of 6T vertebra which was immobilized with a Wilson laminectomy plate bolted to the 5T and 8T spinous processes.

esses of the 11T-12T vertebrae. This resulted in a pronounced kyphosis, undoubtedly due to ruptured ligaments, pain from pressure, and trauma to the cord.

Treatment consisted of a dorsal laminectomy at 11T-12T to remove the bone fragment pressing on the cord. Stainless steel plates, one 7.0 cm. and the other 12.5 cm. in length, were placed on each side of the spinous processes of the involved vertebrae (fig. 16). These plates were secured to the 12T and the 9T spines by means of Wilson laminectomy bolts. Airecast splintage, extending from the shoulder to the flank regions, was used on the abdomen.

The animal died 12 hours following surgery, apparently because of its poor physical condition, spinal shock, and possible interference with respiration caused by the extensive casting.

The method of spinal reduction undoubtedly would have been satisfactory; however, because of the duration of the injury, the animal's eventual recovery was in question.

DISCUSSION OF CLINICAL CASES

Of the three cases presented, two terminated successfully and the other dog might have recovered if it had survived the surgery. It can thus be stated that a select group of spinal fractures can be corrected under some of the following conditions:

1) The animal must have surgical re-

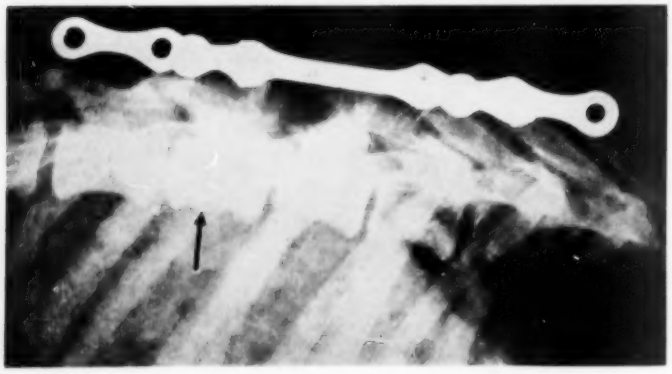

Fig. 16—Spinograph of clinical case 3 showing a compression fracture of 12T vertebra (arrow) with bone plates bolted to 12T and 9T spinous processes.

pair as soon as possible after the injury, preferably within a few hours.

2) The fracture must have only a slight degree of vertebral displacement; a severed spinal cord has little chance of repair. Therefore, compression fractures have the most favorable prognosis.

3) If a bone fragment is causing pressure on the cord, it should be removed by means of a hemilaminectomy or a dorsal laminectomy.

4) Immobilization of the fractured vertebrae should be accomplished by means of bone plates and bolts and, if the dog is large, by additional external splintage.

5) Supportive aftercare is needed to maintain defecation, urination, and good nutrition; to avoid decubital ulcers, and to combat secondary infection.

SUMMARY AND CONCLUSIONS

1) Methods of spinal immobilization by means of bone grafts were studied in 14 dogs. Various types of bone grafts, with and without efforts of spinal immobilization, were employed. As far as a "take" was concerned, there was little difference in onlay, wedge, and H-type grafts. However, the secondary bone proliferation in the wedge-type grafts gave the best immobilization and fusion of vertebrae. Some additional support to the back, while the graft was being established, was desirable for best results. However, the profound immobilization of the vertebrae accomplished by the additional placing of bone plates and bolts to the spinous processes was not satisfactory for a bone graft. Apparently some slight motion is desirable.

2) Vitallium and stainless steel plates placed on 4 dogs with normal vertebrae, by means of screws and bolts of the same material, were well tolerated by the tissue.

3) Methods of spinal fracture immobilization were tried in 8 dogs which were subjected to simulated spinal fractures by surgically removing the bony laminae of the spinal canal and severing the intervertebral disc structures at a certain disc level. The spinal meninges and cord were the only support of the spinal column at this point. The results when plates and bolts were applied to spinous processes, were excellent except in dogs which were too young or too large, so that stress applied to the plate might break the spinous process. Additional wiring of the involved spinous processes, as well as additional external splintage, was helpful. Plates and screws were entirely unsatisfactory.

4) Of three clinical cases of compression fractures of the spinal column, two terminated successfully. Indications for surgical correction for spinal fractures are outlined.

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Studies on Experimental Canine Interstitial Nephritis
II. An Attempt at Functional-Morphological Correlation in Damaged Kidneys

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DUE TO THE widespread incidence of canine interstitial nephritis and the need for more information on the functional-morphological development of the lesions, an attempt was made to devise an approach to this type of study. A series of experiments undertaken to develop such a method has been described.[1] In that study, it appeared that lithium carmine, and to a lesser extent *Staphylococcus aureus* toxin, showed some promise as tools for inducing the interstitial lesion in rather definite increments. It was also found that dogs with surgically explanted urinary bladders made promising chronic preparations and allowed the nephrotoxic agent to be injected into one renal artery, thus retaining the opposite kidney as a control.

With this in mind, a series of animals was used to test each of these agents. These animals were studied by means of clinical urinalysis, urinary clearance tests, and postmortem examinations in an attempt to correlate renal functional changes with any change in kidney structure.

METHOD

Eight female dogs were used to test the effect of lithium carmine * and *Staphylococcus aureus* toxin.** These animals ranged in weight from 30 to 60 lb. and were separated into two groups, one group of 5 used to test lithium carmine and the second group of 3 used to test the toxin.

The urinary bladders of these animals were explanted to the ventral wall of the abdomen to facilitate the separate collection of urine from each kidney.[1] These operated dogs were kept in individual box stalls, and the floor of these stalls was covered with straw. An elevated lattice platform was placed on the floor to be used for resting and sleeping. The straw and platform helped to minimize skin irritation from urine.

Renal clearance tests consisted of two pretreatment and two post-treatment trials of inulin, sodium p-aminohippurate (PAH), and endogenous creatinine clearances. For this purpose the unanesthetized animal was restrained in a modified Pavlov-type sling, and a special device was used for collecting individual urine samples from the left and right kidney (fig. 1). These urine samples were collected over 15-minute periods and measured by use of a 50-ml. graduate.

Injection of the test substance was made by continuous intravenous infusion through a polyethylene catheter † placed in the external jugular vein. The injection was made by the mercury displacement method (fig. 2) and was performed in two phases. The concentration of inulin remained constant during both injection phases, but the sodium p-aminohippurate was administered at two different concentrations, the higher concentration being used to test maximum tubular excretion. Three urine collections were made at each p-aminohippurate blood level.

Blood samples ranging from 10- to 15-ml. in volume were drawn at the midpoint of each urine collection period. The blood was drawn into a heparinized syringe for these samples.

Injections of the nephrotoxic substance were made directly into one of the renal arteries during repeated laparotomies. The injection procedure has been described in a previous publication.[1] One change was made, however, in the procedure used in the 3 animals injected with Staphylococcus toxin. In these animals, the needle was inserted into the aorta near the renal artery and then directed into the lumen of that artery. This was suggested by the technique of Chinard[2] and appeared to minimize damage to the renal artery and limit the amount of periarterial adhesions.

Urine samples were taken before treatment and periodically during the experiment. These were then analyzed for specific gravity, urine pH, albumin, and urinary sediment.

A systematic necropsy was performed on each animal. All animals were electrocuted and after a rapid examination of the urogenital system, sections were taken from each kidney *in situ*. This was followed by the removal of both kidneys, and the section of vena cava and aorta supplying them, as a unit. This allowed a careful examination of each kidney and its blood supply. If there was

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The authors wish to express their appreciation to Dr. J. H. Sautter for taking the photomicrographs included in this study. They also want to thank Dr. W. M. Dickson for his aid in the statistical evaluation of the clearance data.

* Hartman-Leddon Co., Philadelphia, Pa. (a 5% solution was used.)

** Method of standardization described previously.

† P.E. 200, Clay-Adams, New York, N. Y.

any question as to patency or other damage, the renal vessels were sectioned along with the kidneys and other tissues.

The ureters of these animals were also examined closely and sections were taken of the liver and spleen of all animals. Additional sections were taken of any other tissues which appeared abnormal on gross examination.

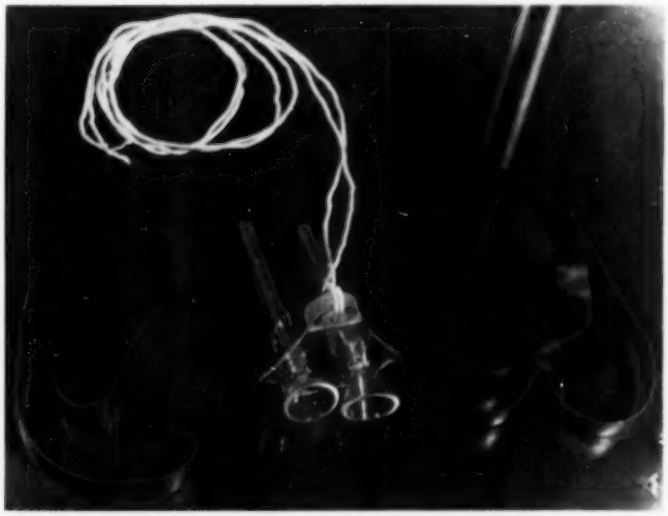

Fig. 1—Cups used for the separate collection of urine samples from left and right kidneys. The cups are placed over ureteral openings, the string is drawn back between hindlegs and then passed on each side of the tail anteriorly to attach to a strap halter. The adjustable rubber straps are passed forward and attached to the same halter.

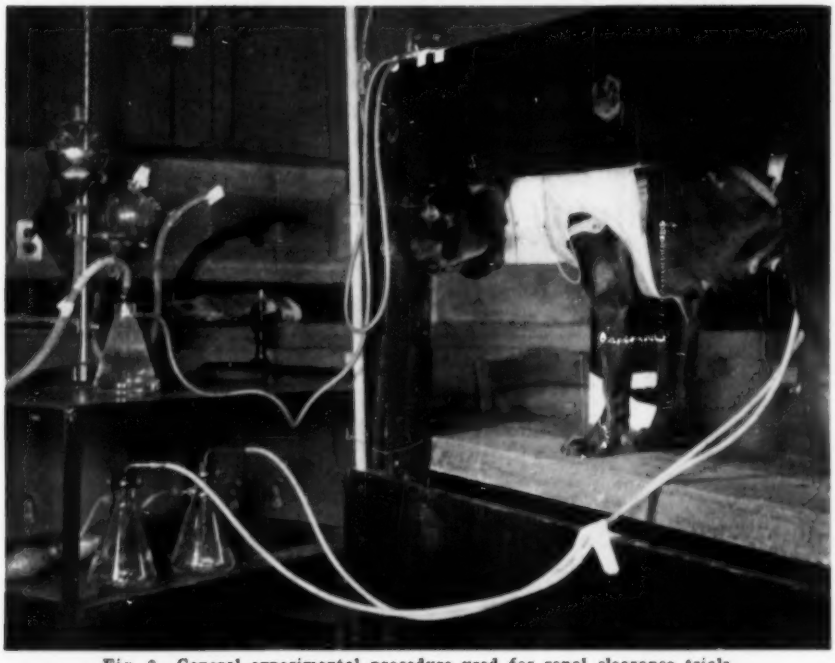

Fig. 2—General experimental procedure used for renal clearance trials. Constant infusion mercury pump is at upper left. Flasks for collection of separate urine samples are at lower left, connected to collection cups strapped to the dog. The dog is restrained by modified Pavlov-type dog sling.

TABLE 1 — Experimental Procedures, and Results of Urinalysis and Postmortem Examination in Dogs Receiving Lithium Carmine and Staphylococcus Toxin

Dog (No.)	Preparation	Substance used	Injection schedule: No. of inject.	Injection schedule: Route	Injection schedule: Dose ml./lb.	Urinalysis	Results of postmortem examination of kidneys
24	Bladder explantation	Lithium carmine *	First (day 1)	Renal artery	0.05	Preinjection urine normal. Exper. kidney following injection: appearance of albumin. Control kidney, albumin (less than experimental).	Animal examined 42 days after first injection. Both kidneys appeared essentially normal.
			Second (day 5)	Renal artery	0.1	Exper. kidney following injection: albumin. Control kidney, albumin (less than experimental.)	
			Third (day 16)	Renal artery	0.1	Exper. kidney following injection: albumin and epithelial cells. Control kidney, albumin (less than experimental).	
25	Bladder explantation	Lithium carmine	First (day 1)	Renal artery	0.1	Preinjection urine normal. Urine normal during interval between injection periods 1 and 2.	Animal examined 29 days after first injection. Both kidneys appeared essentially normal.
			Second (day 10)	Renal artery	0.1	Exper. kidney following injection: appearance of albumin; lower sp. gr. than control. Control kidney, albumin (less than experimental).	
			Third (day 17)	Renal artery	0.1	Exper. kidney following injection: albumin, epithelial cells and hyaline casts; lower sp. gr. than control. Control kidney, albumin (same as exper.).	
26	Bladder explantation	Lithium carmine	First (day 1)	Renal artery	0.18	Preinjection urine normal. Urine normal during interval between injection periods 1 and 2.	Animal examined 57 days after first injection. Both kidneys appeared essentially normal.
			Second (day 8)	Renal artery	0.18	Exper. kidney following injection: albumin (trace), epithelial cells and leukocytes. Control kidney normal.	
			Third (day 19)	Renal artery	0.18	Exper. kidney following injection: albumin (trace) epithelial cells and leukocytes. Control kidney normal.	
27	Bladder explantation	Lithium carmine	First (day 1)	Renal artery	0.3	Preinjection urine normal. Exper. kidney following injection: albumin, epithelial cells and hyaline casts. Control kidney, albumin.	Animal examined 21 days after first injection. Exper. kidney contracted, and cortex decreased in thickness; entire kidney stained red; area of apparent infarct near one

			Second (day 9)	Renal artery	0.3	Exper. kidney following injection: increased epithelial cells and occasional hyaline casts; lower volume and sp. gr. than control. Control kidney, albumin and epithelial cells.	pole; many areas of peritubular connective tissue infiltration; scattered foci of lymphocytes; some convoluted and collecting tubules dilated. Control kidney essentially normal.
28	Bladder explantation	Lithium carmine	First (day 1)	Renal artery	0.3	Preinjection urine normal. Exper. kidney following injection: albumin and epithelial cells. Control kidney, albumin and epithelial cells (less than exper.).	Animal examined 41 days after first injection. Exper. kidney: scattered areas of cortex lighter than the rest, entire kidney stained red; few foci of lymphocytes; large degree of connective tissue infiltration and tubular dilation. Control kidney essentially normal.
			Second (day 9)	Renal artery	0.3	Exper. kidney following injection: epithelial cells and hyaline casts. Control kidney normal.	
			Third (day 16)	Renal artery	0.3	Exper. kidney following injection: albumin, epithelial cells and hyaline casts; lower volume and sp. gr. than control. Control kidney, albumin and epithelial cells (much less than exper.).	
29	Bladder explantation	Staph. toxin	First (day 1)	Renal artery	1×10^{-3}	Exper. kidney, appearance of albumin, epithelial cells and leukocytes. Control kidney, albumin (same as exper.), epithelial cells (much less than exper.).	Animal examined 17 days after first injection; developed symptoms of encephalitis. Exper. kidney adhesions of anterior pole to surrounding structures; grey in color; replacement of large areas of cortex and medulla with connective tissue; rest of kidney shows focal areas of lymphocytic infiltration. Control kidney essentially normal.
			Second (day 7)	Renal artery (thru aorta)	1×10^{-4}	Exper. kidney following injection: albumin and epithelial cells; larger volume and lower sp. gr. than control. Control kidney, albumin (same as epithelial cells) (less than exper.).	
30	Bladder explantation	Staph. toxin	First (day 1)	Renal artery (thru aorta)	1×10^{-3}	Exper. kidney following injection: albumin, epithelial cells and leukocytes. Control kidney, albumin (less than exper.).	Animal examined 34 days after first injection. Exper. kidney, some dilation of convoluted tubules, hyaline casts present. Control kidney, hyaline casts in convoluted tubules.
			Second (day 16)	Renal artery (thru aorta)	1×10^{-4}	Exper. kidney following injection: albumin. Control kidney, albumin (less than exper.).	
32	Bladder explantation	Staph. toxin	First (day 1)	Renal artery	1×10^{-4}	Exper. kidney following injection: albumin and epithelial cells. Control kidney, albumin (less than exper.).	Animal examined 46 days after first injection. Exper. kidney, some dilation of convoluted tubules; granular casts in collecting tubules. Control kidney, some dilation of convoluted tubules (less than experimental); hyaline casts in a few tubules and some areas of congestion.
			Second (day 9)	Renal artery (thru aorta)	1×10^{-4}	Exper. kidney following injection: albumin, epithelial cells and hyaline casts. Control kidney, albumin (less than exper.), few hyaline casts.	

* Hartman-Leddon Co., Philadelphia, Pa. (a 5% solution was used).

Sections of all tissues were stained with hematoxylin-eosin and, in a few cases, kidney sections also were stained with azocarmine.

RESULTS

A summary of the procedures and results can be found in tables 1, 2, and 3.

Series Injected with Lithium Carmine.—The dogs injected with carmine can best be considered as two groups. Those dogs given low level doses of the dye will be described in one group and those given the higher dosage in the second group.

The 3 dogs treated with the lower dosage levels showed much the same results on urinalysis. Albumin was present in the urine after either the first or second injection and remained until termination of the experiment in each case; however, it decreased in the days following the injection. It was seen in the urine from both kidneys of dogs 24 and 25 but was always in higher concentration in the samples from the treated kidney. Epithelial cells appeared in the urine from the treated organ after the second or third injection in all 3 dogs, and these were joined by hyaline casts in dog 25 and leukocytes in dog 26. Dog 25 showed a relative decrease in the specific gravity of the urine from the treated organ after treatment, while dog 24 showed a relative increase in urinary concentration (table 1).

As a group, the dogs treated with the lower dosage level of lithium carmine showed no significant change in the rate of glomerular filtration or of renal plasma flow (table 2) after treatment. This was true of both the total (right kidney plus left kidney) clearance values and the injected/noninjected kidney clearance ratios. However, the total postinjection renal clearance values of dog 24 did show a significant * decrease in the glomerular filtration and renal plasma flow when compared to the preinjection values (Student's "t" test). Also, there were indications of a relative decrease in filtration rate and renal plasma flow of the injected kidney of dog 24 and in the filtration rate of dog 26. However, a relative increase in these values was indicated for the injected organ of dog 25. The filtration fraction of each of these animals decreased following treatment.

The TmPAH ** values determined for these 3 dogs, as well as those determined on the rest of the animals in the experiment, were higher than values determined by other authors and showed large variation between samples from the same dog. No attempt was made to analyze them statistically.

The extrarenal lesions seen in these animals consisted of a generalized thickening of the intestinal wall and hypertrophy of the Peyer's patches and mesenteric lymph nodes. All 3 dogs had adhesions in the region of the injected renal artery, but the vessels appeared undamaged.

Dogs 25 and 26 each had a small carmine stain on the internal surface of the renal pelvis but, other than this, no essential difference could be seen between the injected and noninjected kidneys of the 3 dogs in this group.

The second group of dogs consisted of 2 animals, dogs 27 and 28, each of which was given 0.3 ml. of lithium carmine per pound of body weight. Albuminuria was again a common aftermath of the injections. This was seen in the samples from both treated and control kidneys and often was joined by epithelial cells in both samples. Hyaline casts appeared in the urine from the injected kidney after the first injection of dog 27 and after the second injection of dog 28. These persisted, although sometimes sporadically, until terminus in each case. Perhaps a more definite indication of kidney damage, however, was the change in the relative ability of the two treated kidneys to concentrate and eliminate water.

The fifth day after the second injection of dog 27, water was given orally and the urine was collected over a 20-minute period. During this period, a volume of 151 ml. (sp. gr. 1.003) was excreted from the control kidney and a volume of 10.4 ml. (sp. gr. 1.001) was collected from the injected kidney. A dilution test run 12 days after this second injection gave the following results. Over a period of 3 hours and 37 minutes, 648 ml. was collected from the control kidney and 64 ml. from the injected kidney. Specific gravity measurements previous to the administration of water were: control kidney, 1.045; experimental kidney, 1.025. Although the specific gravity then was measured every 15 minutes for the next 3.5 hours, neither kidney was capable of concentrating the urine beyond a specific gravity of 1.018.

A dilution test also was run 28 days after the third injection of dog 28. During

*P = 0.01 (creatinine); P = 0.05 (PAH); ** maximum tubular excretory capacity for para-aminohippuric acid.

TABLE 2—Averages of the Total Clearance Values (Right Kidney Plus Left Kidney) of Each Animal Before and After Treatment, and the Ratio of the Treated Kidney Versus Non-treated Kidney Renal Clearance Values (Total Clearance Values Given in Milliliters per Minute per Square Meter of Body Surface)

Animal No.	Creatinine		Inulin		P aminohippuric acid		C_{Cr}
	Total	Cont. kid. / Treat. kid.	Total	Cont. kid. / Treat. kid.	Total	Cont. kid. / Treat. kid.	C_{PAH} Filtration Fraction
				CARMINE TREATED			
24 before	106	1.05	76	1.02	340	0.82	31
24 after	82	1.15	62	1.07	270	0.86	30
25 before	75	1.10	54	1.01	300	1.30	25
25 after	81	0.93	66	0.86	370	1.1	22
26 before	78	0.61	60	0.60	310	0.77	25
26 after	63	1.01	73	0.89	310	0.90	20
27 before	74	0.71	81	0.82	370	0.78	20
27 after	59	8.80	62	9.60	210	10.3	28
28 before	86	0.94	61	0.98	350	1.08	25
28 after	55	8.30	45	7.70	220	10.5	25
				STAPHYLOCOCCUS AUREUS TOXIN TREATED			
30 before	77	0.98	51	1.02	270	0.85	29
30 after	53	1.36	46	1.10	120	1.30	44
32 before	93	0.93	46	1.02	180	1.15	52
32 after	83	1.16	50	1.20	180	1.00	46

C_{Cr}—clearance, creatinine; C_{PAH}—clearance, para-aminohippuric acid.

a 2-hour and 40-minute collection period, the control kidney excreted 275 ml. of urine while the injected kidney excreted 115 ml. At the end of three hours, the specific gravity had returned to 1.010 in the sample from the injected kidney and 1.023 in the sample from the control kidney.

A significant difference could be shown between the preinjection and the postinjection clearance values of these 2 dogs (table 2). The total amount of glomerular filtration and renal plasma flow of both kidneys decreased and the relative (and actual) ability of the injected kidney to perform these functions decreased markedly. This was confirmed by an analysis of covariance in which postinjection values were adjusted for preinjection differences. The total (left plus right kidney) filtration fraction showed an increase after injection of dog 27, but no change in filtration fraction was noted in dog 28. Although as previously stated, the data on tubular excretion were not analyzed statistically, there was a definite indication of a relative and actual decrease in the tubular function of the injected kidneys of these animals.

Extrarenal lesions in these 2 animals consisted of the same intestinal thickening and lymphatic hypertrophy and adhesions near the site of arterial injection. The only other lesion noted was a small thrombus, approximately 1 cm. in diameter, located in the right atrium of dog 28.

On gross examination, the control kidneys of both dogs were essentially normal and the injected kidneys of both were stained red throughout their parenchyma. However, except for this stain, the kidneys from the 2 dogs differed quite markedly. The injected kidney of dog 27 was contracted and a lighter color than the control organ. Also, near one pole a dark-staining circumscribed area occupied about 1 cm. of space in the medulla. The injected

TABLE 3—Pretreatment Renal Clearance Values Determined on the 5 Dogs Treated with Lithium Carmine

Test substance	Number of observations *	Mean glomerular filtration rate ml./m²		Renal plasma flow ml./m²	
		Of dogs tested	Of individual kidneys tested	Of dogs tested	Of individual kidneys tested
Creatinine	35	89 ± 25**	44 ± 14	.....	.....
Inulin	33	67 ± 18	34 ± 9.2	.....	.....
P-amino-hippuric acid	21	.....	.....	356 ± 87	178 ± 47

* This represents the total number of observations made on the 5 dogs. The number of observations made on individual kidneys would be twice this.

** Standard deviation.

kidney of dog 28 was approximately the same size as the control organ. The only major difference between the two kidneys of this dog on gross examination was the lighter color of the injected kidney's cortex.

Microscopic examination further emphasized the structural changes in the treated kidneys of these dogs. The circumscribed medullary lesion in the kidney of dog 27 consisted of connective tissue, and the cortex above this area showed a few areas of complete necrosis suggesting an infarct. Sections of the renal artery were examined and in one of these sections the continuity of the endothelium appeared to be broken and erythrocytes were present in the subendothelial tissue.

Sections taken from the injected kidney of dog 27 outside of the circumscribed lesion described above were uniform and quite different in character from those taken within this lesion (fig. 3). Some of the tubules were dilated with atrophic epithelia and a large number of tubules was seen to contain epithelial cells in various stages of degeneration and their lumens were filled with cellular debris and casts. Foci of lymphocytes were scattered throughout the cortex, disposed in peritubular, periglomerular, and perivascular accumulations. Some connective tissue infiltrations were noted on differential staining. The glomeruli, in a few cases, showed a hypertrophy of their epithelial cells and increased thickness of the basement membranes, but the great majority appeared normal.

The injected kidney of dog 28 showed more chronic lesions (fig. 4). Mononuclear cell infiltrations were less prominent and much more connective tissue was seen, in some places replacing tubules. There was a greater number of tubules showing extreme dilation and the normal epithelial cells of these tubules had been replaced with atrophied or new immature cell types. The control kidneys of dog 27 and dog 28 both appeared essentially normal.

Series Injected with Staphylococcus Toxin.—The first dog in this series injected with the Staphylococcus toxin became very ill following the second injection, developing an elevated body temperature and depression. The eighth day after this in-

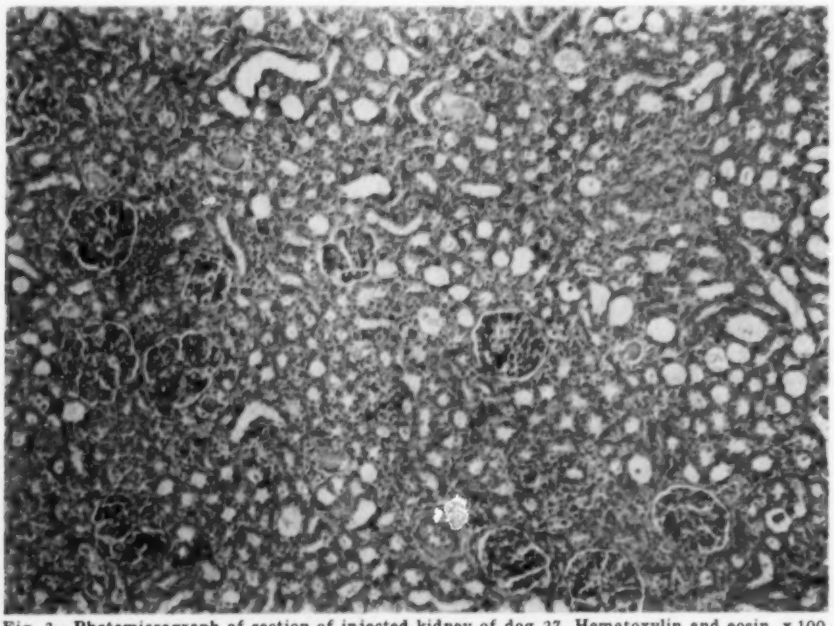

Fig. 3—Photomicrograph of section of injected kidney of dog 27. Hematoxylin and eosin. x 100. Section from the cortex at opposite pole from circumscribed lesion. Notice the area of lymphocytic infiltration in upper left portion of photograph and dilation and epithelial atrophy in many tubules. Hyaline casts are present in some tubules. Some increase in perivascular connective tissue can be seen.

jection, symptoms of encephalitis appeared and the animal was destroyed two days later.

On necropsy, the liver showed chronic passive congestion. The lung also was congested and there was some evidence of edema, proliferation of the bronchial epithelium, and interstitial pneumonia. The control kidney appeared essentially normal, but the injected kidney exhibited the usual adhesions in the area of the pelvis and also was attached by adhesions of its anterior pole to surrounding structures. On gross sectioning of the kidney, this area near the anterior pole was seen to be circumscribed and gray.

The microscopic picture was varied. Sections cut from the region of the anterior pole showed partial to complete necrosis of the parenchyma, while sections taken from the rest of the kidney exhibited areas of normal kidney adjoining areas with connective tissue infiltration and replacement of the tubules. These affected portions of the kidney also contained scattered foci of lymphocytes.

Urinalysis following the injection of toxin into this series of animals showed albumin and epithelial cells to be present in the urine from the injected kidneys. A decrease in volume and specific gravity accompanied these changes in dog 29, and hyaline casts were noted in the urine of dog 32.

Renal clearances determined on dogs 30 and 32 indicated a decrease in the total filtration rate and renal plasma flow of dog 30, and relative decreases in the filtration rates of the injected kidneys of both animals and in the plasma flow of dog 30. The total filtration rate and plasma flow for both kidneys of dog 32 gave no indication of definite change, and the renal plasma flow in the injected organ showed a relative increase. The filtration fraction, following treatment, showed a decrease in one of these dogs and an increase in the other.

Postmortem examination of dogs 30 and 32 gave no indication of extra-renal lesions, but casts were present in the tubules of both the experimental and control kidneys.

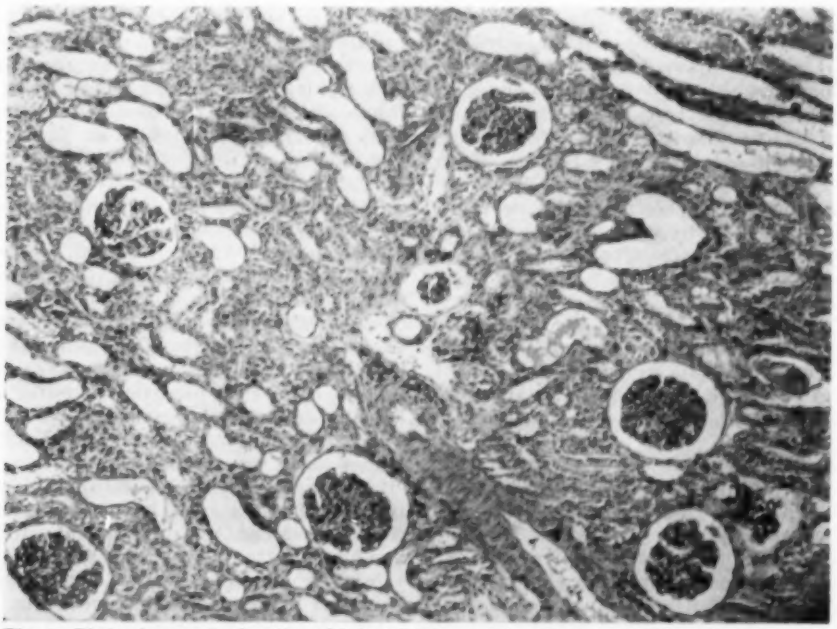

Fig. 4—Photomicrograph of section of injected kidney of dog 28. Hematoxylin and eosin. x 100. Typical section from cortex showing replacement of some tubules with connective tissue and destruction or dilation of others. Notice periglomerular connective tissue in some areas and perivascular accumulation of lymphocytes in lower center of photograph. Increased separation can be seen between glomeruli and capsules.

There was also a moderate degree of dilation of the convoluted tubules in the two experimental kidneys. The convoluted tubules of the control kidney of dog 32 also were dilated, but this dilation, and also the number of casts in the control kidneys of the 2 animals, were less than that seen in the injected organs.

DISCUSSION

An attempt was made to produce interstitial nephritis experimentally in dogs by the injection of either lithium carmine or *Staphylococcus aureus* toxin directly into the renal artery. This was done on female dogs with surgically explanted urinary bladders to facilitate separate assessment of the functional ability of the injected and noninjected kidney before, during, and after treatment. Renal function was determined by pre- and post-treatment renal clearance trials and also by periodic clinical analysis of the urine throughout the experiment. The results of these functional tests were then studied in relation to the morphological changes seen at the time of necropsy.

Lithium carmine was administered at two dosage levels. Three animals were injected with the lower dosage. The presence of albumin, epithelial cells and, in one case, casts in the urine from the treated kidney following injection indicated that the dye was having some effect. Although there were some further indications of change in renal function as shown by the clearance trials, with the exception of the total glomerular filtration and renal plasma flow in dog 24, the differences did not prove statistically significant. However, the filtration fractions calculated for each of these animals showed a decrease after treatment.

Necropsy of the injected and the control kidneys of these dogs showed no apparent difference, both kidneys appearing essentially normal.

The 2 animals injected with a larger dose of lithium carmine showed a more profound effect. Relatively large numbers of hyaline casts were seen in the urine samples from the treated kidney, and the volumes and specific gravities of these samples were markedly decreased in comparison with samples from the control organs. Renal clearance tests gave further indication of kidney damage showing a significant decrease in the ability of these animals to filter urine, and also a significant decrease in renal plasma flow. This decrease in renal function applied to both the total activity of both kidneys after treatment and to the relative activity of the treated organ versus the control organ. The filtration fractions increased following treatment of one of these dogs and showed no change for the other animal.

Total glomerular filtration rate and renal plasma flow have been calculated for the dog by a number of workers. Houck,[3] using 75 mongrel dogs, found the glomerular filtration rate to be 84.4 ± 19.1 ml./m² (creatinine), while the renal plasma flow was 266 ± 66 ml./m². Corcoran and Page [4] made 77 observations on 7 female dogs and obtained a glomerular filtration rate of 69 ± 14.8 ml./m² (inulin) and a renal plasma flow of 250 ± 61 ml./m². Moustgaard [5] tested 118 dogs and found a glomerular filtration rate of 87 ± 16 ml./m² (creatinine) and a renal plasma flow of 260 ± 29.

Examination of the total (left plus right kidney) values achieved by the collection procedure used in this experiment shows comparable deviations from the mean considering the small number of animals tested and the added error one would expect from collection of individual kidney urine samples (table 3). The glomerular filtration values determined by endogenous creatinine closely approached those found by Moustgaard and by Houck while filtration determined by the inulin method was close to that determined by Corcoran and Page. The mean renal plasma flow calculated here was considerably higher, however, than that determined by these authors. It is difficult to explain the difference, although a possible explanation might be the small number of animals tested in this series plus the fact that they were of approximately the same weight. The series used thus constituted a less random population sample.

The disagreement between the TmPAH values as determined here and those of other authors may be due partly to higher renal plasma flow rates, but the wide range can be explained only by error in technique due possibly to the large dilution of low volume single kidney samples, which would tend to magnify any collection errors.

On postmortem examination, the treated kidneys of dogs 27 and 28 looked much like kidneys seen in field cases of interstitial nephritis. The injected organ of

dog 27 resembled the subacute stage, while the injected kidney of dog 28 resembled the chronic stage of the interstitial lesion. (The control kidneys of both of these animals appeared essentially normal.)

In a previous article,[1] an experiment was described, among others, in which the dog went through the same general experimental procedure as was described for the above 5 dogs. No renal clearance trials were run. That dog received three injections of lithium carmine at three different dosage levels (0.04 ml./lb., 0.15 ml./lb., and 0.3 ml./lb.). These doses range between the minimum and maximum doses given the 5 dogs in this experiment (table 1).

The urine from the injected kidney of that dog contained casts and showed a relative increase in volume and decrease in specific gravity. Also, on postmortem examination, sections of the injected kidney closely resembled those from the kidneys of animals with clinical cases of acute interstitial nephritis. Thus, in the view of the writers, this dog represents a useful contrast with dogs 27 and 28 in the present series.

It then would appear that lithium carmine may be capable of producing the lesions resembling those seen in clinical cases of interstitial nephritis in the dog. If this can be done, the functional and morphological development of the renal lesion can be studied under controlled conditions. It would be possible also to test the efficacy of various treatments, such as cortisone, for the prevention of the interstitial reaction. The small number of animals treated with effective dosages of the carmine did not lend itself to ideal statistical evaluation, and the necessity for further corroboration of these results is obvious.

Only 3 dogs were treated with the Staphylococcus toxin, one of which died during the experiment, and the results were inconclusive. It would appear, however, that the toxin is much more difficult to control in attempts to produce chronic kidney damage.

SUMMARY

1) An attempt was made experimentally to produce interstitial nephritis in dogs for the purpose of studying functional-morphological change relationships.

2) Two nephrotoxic substances previously chosen for this purpose were each injected unilaterally into the renal artery of dogs with surgically explanted urinary bladders as follows: *(a)* lithium carmine—5 dogs; *(b) Staphylococcus aureus* toxin—3 dogs.

3) Clinical urinalysis gave only an approximate indication of the amount of damage done by injection, consisting of the presence of albumin, epithelial cells and, in some animals, leukocytes and casts in the sample from the injected kidneys. Samples from the control kidneys varied from those containing albumin and epithelial cells to samples showing no changes. Casts, when present, appeared to be the best indication of damage.

4) Two of the animals which were injected with higher dosage levels of lithium carmine showed lesions in the injected kidney resembling clinical interstitial nephritis. The urine from the injected kidneys of these dogs showed a relative decrease in volume and specific gravity.

5) The above 2 animals also showed a decrease in filtration rate, renal plasma flow, and TmPAH.

6) Dogs injected with Staphylococcus toxin showed indications of a relative change in the structure and function of the injected kidneys, as compared to the noninjected kidneys. The results of this experiment, however, were inconclusive.

7) Pretreatment glomerular filtration values in animals with bladder explantation compared favorably with those reported in nonsurgical animals in other laboratories. The renal plasma flow values were higher than those obtained by other authors, and measures of tubular excretion were variable.

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Flexor Spasm Syndrome in the Carnivore
I. Review of Its Occurrence in the Literature as "Canine Chorea"

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THAT ABNORMAL involuntary activity is frequently a residual of distemper has been known to veterinarians for over 100 years. It is the purpose of this series of articles to review understanding of such hyperkinesia, to present a unique occurrence in a large member of the feline species, and to summarize clinical observations and pathological changes found in this laboratory to date.

In 1817, Blaine [1] recognized "spasmodic twitching" in his excellent description of the disorder then known to veterinarians as "distemper":

> The distemper is very frequently attended with convulsions: it is also now and then preceded by them. These convulsive affections are of two kinds: one is a simple paralytic affection, which, in addition to the weakness, frequently leaves a spasmodic twitching also in one or more of the limbs for life. The other is a perfect fit. . . .

A remarkable characteristic of the spasmodic twitching was first noted in 1862 by Chauvau,[2] who stated that the hyperkinesia persisted below transection of the spinal cord. Chauvau's observation, an example of serendipity, was made during an attempt to obtain lymph from the thoracic duct of a dog. The animal survived an attempt to destroy it by transection of the cervical cord because hyperkinesia affecting thoracic musculature provided respiration enough to sustain life. In 1869, Carville [3] confirmed Chauvau's observation and during the next 20 years eight articles appeared in the literature to demonstrate the interest aroused in this phenomenon (table 1).

In 1870, under the title "Choreiform Movements of Dogs," Le Gros and Onimus [4] published the results of extensive physiological research upon the spasm syndrome,* again confirming the persistence after section of the spinal cord, demonstrating diminution in amplitude following section of pertinent dorsal roots, and its abolition following section of pertinent ventral roots or section of the motor nerves. They introduced data on pharmacology, observing that the effect of chloral hydrate given rectally was to diminish the amplitude and to increase the frequency of the activity. Electrophysiological analysis was begun with the observations that amplitude was decreased if the spinal cord was stimulated above the level of activity and increased if stimulated from below, and that the activity persisted unaffected during the tetany attending stimulation of the sciatic nerve.

Since the residual twitchings of the syndrome were abnormal involuntary muscular activity, the interest of clinicians was stimulated by the possibility that it was an animal analogue of chorea in man, hence offering the possibility for experimental analysis of the latter. In England, this interest generated seven articles (table 1) two years after publication of the Le Gros and Onimus' work, beginning with an article by Hughlings Jackson [5] in 1872 describing his observations on "a case of chorea in a dog."

Even at this time the use of the word "chorea" to designate differing dyskinetic phenomena was confusing the literature and delaying understanding. In a clinical discussion on chorea, Gowers [6] reported his awareness of this terminological danger by writing (p. 277): "I have preferred not to use the term posthemiplegic chorea in speaking of any of these cases for two reasons: first, I think the term tends to confuse together several easily distinguishable

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* This terminology serves the need for descriptive definition; "twitch" and "jerk" as alternative designations occur in different contexts. A "flexor" characteristic of the activity, not previously described, is developed in Part III of this series. "Choreoid" and "tremor" activities are quite different in manifestations (vide infra) and "myoclonus" or "myorrhythmia" are in loose usage.

varieties of movement, which may ultimately be found to depend upon pathological processes of different kinds or in different localities; secondly, the word 'chorea' . . . were better not transferred to a symptom."**

Although Jackson used the term "chorea," stating that it was not uncommon in young dogs and almost invariably followed distemper, he did make a phenomenological distinction: "The movements are very different from those in chorea of the human subject . . . there are chiefly rapid repetitions of one movement rather than, as in the human subject, successions of different movements." He gave an excellent description of the syndrome and indicated his broad interest in neurological phenomena by inviting notice of instances of chorea in the cat and requesting "references to any American, French, or German works which dealt with the clinical history and pathology of dogs, cats, horses, sheep, etc." Inexplicably, he stated that "there is no movement in sleep," apparently missing what is one of the most remarkable features of the syndrome (*see* Parts II and III).

At the time of his publication, Jackson could provide no experimental observations but Gowers[7] was able three years later (1875) to confirm, for the third time, that the activity persisted following transection of the spinal cord. As a result of his observation, he contributed to speculation concerning the cause of chorea by suggesting that this type of dyskinesia might actually be independent of the cerebrum. In 1877 he and Sankey,[8] describing further studies on the effects of spinal cord transection, made the observation that etherization abolished the activity. They also described the pathological changes in the central nervous system of an affected animal for the first time, noting perivascular infiltrations and pathological changes in neurons and indicating some concern whether the latter might not have resulted from an "overaction" of the affected motor units.

In 1879, Putnam[9] furthered the existing confusion of "chorea" and the spasm syndrome of carnivores by describing "chorea" in a kitten and observing that, in this specimen, activity did not persist following section of the spinal cord and, also, that there were no pathological changes to be seen.

Hadden[10] in 1883 described pathological changes similar to those observed by Gowers and Sankey. He continued heuristic efforts to link the syndrome with some recognized human disorder, disagreeing that the phenomena were like chorea and asserting that they were related to spasmodic torticollis. German workers began to publish articles on this subject and in 1885, Quincke[11] again described the effects of transection of the spinal cord, of ether, and of chloral hydrate; he added that morphine produced no change in activity.

Horsley[12] made an extensive experimental study of the pathology associated with the syndrome which was published in 1886. He reiterated the differences between it and chorea in man and commented on how frequently systemic anemia appeared in affected animals. He con-

TABLE 1 — Chronology of Publications on the Flexor Spasm Syndrome

1860	1900	1930	
1	1	1	
2—Chauvau	2	2	
3	3	3	
4	4	4	
5	5	5	
6	6	6	
7	7	7	
8	8	8	
9—Carville	9	9	
1870—Legros, Onimus	1910	1940	
1	1	1	
2—Jackson	2	2	
3	3	3	
4	4	4	
5—Gowers	5	5	
6	6	6	
7—Gowers, Sankey	7	7	
8	8	8—McGovern *et al.*	
9	9	Traeger	
1880	1920	9—Innes	
1	1	1950—Koprowski *et al.*	
2	2	McGovern *et al.*	
3—Hadden	3	1	
4	4	2	
5—Quincke	5	3	
6—Horsley	6	4—Turbes	
7	7	5	
8	8	6	
9	9	7	
1890		8	
1		9	
2			
3—Wood			
4			
5			
6			
7			
8			
9			

** The author has attempted to distinguish between hyperkinesias by a graphic method in animals (Graphic Expression of Cinematographic Records of Animal Dyskinesia. J. Comp. Neurol., *89*. [1948]: 1–19) and in man (The Graphic Study of Ballism and Related Hyperkinesia. J. Neuropath. and Exptl. Neurol., *11*. [1952]: 300–316).

firmed that the activity persisted following section of the spinal cord, demonstrated that it was abolished by section of pertinent motor nerves, and showed that stimulation of the sciatic nerve produced an increase in frequency and a decrease in amplitude. He saw pathological changes similar to those described by Gowers and Sankey and also changes "as of the nature of disseminated sclerosis" (an observation preceding by 44 years that of Perdrau and Pugh [13] in 1930).

In 1893, Wood [14,15] began contributions to the knowledge of the syndrome from the Americas. He confirmed, now for the seventh time, that the activity persisted following transection of the spinal cord; he added the significant observations that the amplitude of the activity was markedly increased following the section and that the frequency became asynchronous between rostral and caudal segments. He contributed the additional observation that the effect of quinine was to diminish amplitude of the activity and finally to abolish it, and that the effect of atropine was to increase amplitude and to bring about a resumption of activity abolished by quininization. In a public presentation, he exhibited a dog in which quinine given orally had been "curative."

The 50 years before the turn of the century, in which 11 publications appeared upon the syndrome, were followed by 55 years during which investigation developed the relation between the various encephalitic phenomena in carnivores and gave only incidental consideration to the twitchings. Partly as an outgrowth of unusual public interest in the distemper of dogs, Laidlaw and Dunkin [16] conducted intensive studies between 1923 and 1931, demonstrating that distemper was in many instances caused by a virus, that ferrets could be used as experimental animals to demonstrate virus passage, and that immunizing procedures could be developed. In 1930, Perdrau and Pugh [13] published an extensive consideration of the pathology of disseminated encephalomyelitis of the dog (the nervous form of canine distemper). They noted the cellular infiltration of vascular and glial elements which had been apparent to earlier writers but gave a specific description of patches of demyelination, observed in 4 of the 14 animals studied, being particularly frequent in cerebellar peduncles and adjacent portions of the cerebellum and brain stem. Their conclusion was that "the virus of distemper played a similar role to that which an acute infection of varying etiology plays in the causation of certain demyelinating diseases of man, such as acute disseminated encephalomyelitis, and possibly also disseminated sclerosis." They apparently overlooked Horsley's observation in 1886,[12] commenting that "the particular feature to which attention is drawn in this paper —demyelination—has not been stressed by any writer with whose work we are acquainted." They commented that distemper was not always apparent in the history of specimens in which the pathological changes of disseminated encephalomyelitis could be demonstrated.

Two publications appeared in 1948 giving the spasm syndrome specific consideration. McGovern, Wyke *et al.*[17] compared the syndrome with parkinsonism in man, described the activity as tremor, and concluded from experimental observations that ablation of areas 6 and 8 of the cerebral cortex was ameliorative. In this same year, Traeger *et al.*[18] observed that during pyrexia, by the use of bacterial vaccine (pyrifer), the activity diminished.

In 1949, Innes [19] published a general study of the pathological changes in canine encephalomyelitis and the essentials of his observations were confirmed in 1950 by Koprowski, Jervis *et al.*[20] as atypical gliosis, giant multinuclear cells, neuron degeneration, and demyelination. Although dyskinetic and specifically hyperkinetic phenomena, appearing as neurological residuals of the disorder, were noted in clinical descriptions, no attempt was made to correlate pathological changes with the occurrence of activity in a specific group of muscles.

In 1950, McGovern, Steel *et al.*[21] made a report on clinical observations and pathological material from several thousands of dogs, which indicated that 50 to 60 per cent of neurological disorders in such animals were associated with previous or coexistent distemper infection but only one third of the dogs which acquired clinical distemper showed signs of neurological deficit; the report further indicated that pathological changes were those of a meningo-encephalomyelitis in which nuclear inclusions were present within the glial cells. Demyelination and cellular infiltration was also noted. While making the excellent

recommendation that the term "chorea," if applied to the canine disease, was misleading and should be discarded, these authors suggested that it be known as the "tremor syndrome," a suggestion which could only contribute to the prevailing confusion in terminology (*see* discussion, Part III).

Turbes [22] recently reported experiments which indicated that the spasm ceases during electrical stimulation of the contralateral motor cortex and of the cerebral peduncles and pyramids; also, the spasm was abolished by ventral and dorsal rhizotomy, by muscle tenotomy, and by bone fracture with a shortening effect on the musculature. He concluded that the physiological mechanism was located in the neural segment.

SUMMARY

A spontaneous spasm syndrome, occurring in the carnivores and frequently following distemper, has been recognized for 150 years. In the literature, the characteristics of the activity by which the syndrome is manifested have been distinguished from those of chorea in man but the syndrome has continued to be so designated, especially among veterinarians, and the recent introduction of the term "tremor" to describe the syndrome tends to further the confusion. Numerous observations have established the autonomy of the activity within the spinal neural segment, independent of the cerebrum, and the influence of numerous drugs upon the syndrome. The pathological physiology of the syndrome is not yet understood, nor have neural pathological changes been demonstrated corresponding to appearance of the activity in specific muscle groups.

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II. Occurrence in a Tiger (Felis Tigris)

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THERE ARE no published records of the occurrence of a spontaneous flexor spasm syndrome (canine chorea) in a feline animal. Such an occurrence in a captive full-grown Bengal tiger "Seton" is the occasion for this report, as a part of a general study of the syndrome.

Observations were made in cages of the lion house and in a hospital cage at the New York Zoological Park.

HISTORY

The specimen (*Felis tigris*) was one of 3 cubs born May 18, 1950, in the third litter resulting

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from brother and sister mating of tigers "Rajpur" and "Dacca" during captivity. A total of 25 cubs born to this pair were healthy in two previous and four subsequent litters. At the age of 3 months, the animal received a routine initial immunizing dose of 10 cc. of feline enteritis vaccine (Lederle) subcutaneously and a second injection was given seven days later. The vaccine was prepared from spleen and liver tissue of artificially infected kittens. Twenty-two other tiger cubs received such vaccine without significant sequelae.

The tigers were displayed in a communal cage until March 22, 1951, when keepers noticed abnormal movements in the left hindleg of this 22-month-old animal. No prior evidence of illness of any kind had been observed in this animal or in its cage mates.

Collaboration was begun between the staffs of the Veterinary Department of the Zoological Park and the Neurological Department of the Columbia College of Physicians and Surgeons, in a combined therapeutic investigation and neurophysiological study, with the purposes of permitting return of the animal to display and of increasing understanding of the disorder.

OBSERVATIONS

Observations made between April 13, 1951, and June 17, 1952, when the animal was destroyed, are presented in chronological sequence:

April 13, 1951 (Cinematography).—Abnormal involuntary activity was apparent, most marked in the left hindleg but present in lower axial musculature and right hindleg. The activity in the hindleg was flexor in direction and simultaneous with activity elsewhere. It was composed of ungrouped movements of almost constant amplitude, which occurred at regular intervals, in delayed sequence and in regular rhythm (42 per minute if the animal was lying, 60 per minute if standing). The effect of each hindleg movement was to jerk the paw from the floor when standing and to jerk thigh and leg against the trunk in the lying posture (graph 1). The animal preferred to lie with the affected leg free rather than beneath it, where it was rubbed against the concrete cage floor. Paresis was present in both hind limbs, more marked on the left, so that in walking the weight-bearing phase was shortened on the left with the limb collapsing during this interval. In the associated activity of walking, the hyperkinesia persisted and distorted the movements which otherwise were normal. The animal's general behavior was not remarkable.

May 26.—Nembutal® sedation was given *per os* and coma ensued for four days during which hyperkinesia was absent. A blood count revealed erythrocytes 6,940,000 per cubic millimeter; hemoglobin, 13.5 Gm.; hematocrit 46 %; leukocytes 11,700 per cubic millimeter (neutrophils 39 %, monocytes 2 %, lymphocytes 55 %, eosinophiles 1 %, basophiles 3 %); plasma specific gravity 1.0278. Boiled cow's milk (20 cc.) was injected, intramuscularly, without hyperpyrexia. The animal was given glucose infusion (400 cc., 5 %) and Metrazol (3 cc. subcutaneously) and recovery from the coma ensued.

August 12 (Cinematography).—Sedation was produced with chloral hydrate (27 Gm.) *per os* at 9 a.m. Deep sleep ensued but he moved his head when prodded and struggled away from a leg snare. Hyperkinesia was present during sleep with a frequency of 18 per minute with amplitude and forcefulness markedly diminished. The animal was weighed (200 lb.) but would not tolerate the rectal thermometer.

September 5.—Wasting was apparent in the left thigh but the status was otherwise unchanged. Spasm frequency was 48 per minute standing and 30 per minute lying.

October 16.—Seconal sedation was administered (2 Gm. *per os* in ¾ grain capsules) *at 8:30* a.m.; rectal temperatures were 98 to 99 F.; he roused and ate at 3:15 p.m.; for the recording at 11 a.m., he required supplementary ether but not thereafter; he was drowsy and unsteady for three days.

October 29.—Seconal sedation (2 Gm.) was given at 8:30 a.m. A rectal temperature was possible at 10:30 a.m. Typhoid vaccine* was given intravenously and the ensuing pyrexia was 104.2 F. (table 1). Ear-flicking reflex (to touch of conchal hairs), corneal reflexes, and hyperkinesia were present during the procedure; also, slight simultaneous activity in both forepaws. The animal was moving and defecated at 8:30 a.m. the following day.

December 17.—Thiantoin** (0.13 Gm. pulvules, 5 pulvules *per os* daily for 7 days, then 6 pulvules daily for 7 days) was administered. Following two weeks of

* Lederle Typhoid-paratyphoid vaccine, 100 million organisms per cubic centimeter (50% *Eberthella typhosa*, 25% *Salmonella paratyphi* A., 25% *Salmonella paratyphi* B).

** 5,5-phenyl thienyl hydantoin (Lilly).

TABLE 1—Temperature Reaction, in a Tiger, to Intravenous Typhoid Vaccine Injections while Under Seconal Sedation

Time of sedation	Vaccine (organisms/cc.)	Cage temp. (F.)	Rectal temp. (F.)*
8:30 a.m.			
10:30			100.6
11:00			100.6
11:40	15 million	76	100.4
12:50 p.m.	25 million		99.4
1:30	25 million	78	100.3
2:05		79	101.4
2:40		80	102.2
2:50	25 million		
3:30			102.8
4:00			102.8
4:05	25 million		
4:45			103.0
7:00			104.2
9:30			103.6

* H. H. Dukes gives the normal temperature for large cats as 100.5–102.5 F., with an average of 101.5 F. (The Physiology of Domestic Animals. 5th ed. Comstock Publishing Co., Inc., Ithaca, N. Y. [1942]: 474.)

medication, forcefulness, amplitude, and frequency of activity were markedly increased (the latter 70 to 80 per minute).

January 13, 1952 (Cinematography).—No medication was given for 13 days. Frequency of spasms was 42 per minute, lying.

January 14.—Thiantoin medication was repeated (6 pulvules *per os* daily for 5 days).

January 20 (Cinematography of Thiantoin Effect).—The cage temperature was 70 F.; the spasm frequency was 75 per minute (range 70–79), lying. Occasional grouped movements, about 5 in almost immediate sequence, were followed by a period of relative slowing in frequency for several movements.

January 21 to 26.—The effect of withdrawing Thiantoin medication was observed (last dose Jan. 19). A slow decrease in frequency of spasms occurred (table 2).

January 28 to February 2.—The effect of tridione† medication (0.3-Gm. capsules, 4 capsules *per os* daily for 3 days then 5 daily for 3 days) was observed; there was a continued slow decrease in frequency of spasms (table 2).

February 3.—Cinematography.

April 18.—Seconal sedation (2 Gm. *per os*) was given. The spasm frequency was 28 per minute after one and a half hours.

April 22.—The spasm frequency was 37; wasting and weakness were marked.

April 23.—Seconal sedation (2 Gm. *per os*) was administered. There was drowsiness at 3 p. m.; hyperkinesia was barely present at 25 per minute. The status was unchanged to April 26 when the animal began to crawl about the cage and, finally, to sit. Frequency of spasms was 30 per minute.

† Trimethadione (Abbott).

TABLE 2—Spasm Frequency in a Tiger During Medication with Thiantoin and Tridione

Date	Medication	Time	Posture	Frequency of spasm (per min.)
1952				
Jan. 14			Lying	42
20	(After 5 days of thiantoin)	4:00 p.m.	Lying	75
21			Standing	78
22			Standing	72
23			Lying	55–61
24		9:00 p.m.	Sitting	49
			Standing	55
25		8:30 a.m.	Lying	57
26			Lying	48
28	Tridione			
29	Tridione		Lying	37
30	Tridione	a.m.	Lying	36
		p.m.	Lying	42
31	Tridione	3:00 p.m.	Lying	36
Feb. 1	Tridione	9:00 a.m.	Lying	33
		4:30 p.m.	Lying	37
2		1:30 p.m.	Lying	37
3			Lying	35
4			Lying	38
7			Lying	37

April 30.—Seconal sedation (2 Gm. *per os*) was given. The animal was comatose at 3 p.m.; the rectal temperature, 100 F.; the weight, 303 lb. The frequency of spasms, on the following day, was 22 per minute when he was still comatose.

May 7.—P–267 medication‡ was administered (table 3). Forcefulness and frequency of spasms were increased; a convulsion occurred from overdosage.

May 19 to June 26.—Attempts to establish Seconal tolerance were inconclusive (*see* discussion).

June 17.—The animal was destroyed by acute experiment. Seconal sedation was obtained with 1.6 Gm. *per os* at 4:30 p.m., June 16, and at 1:30 p.m., June 17, sup-

‡ P–267 is the designation used by Pitman-Moore Company, Indianapolis, Ind., for 4-n-butoxy-B-(1-piperidyl) propiophenone hydrochloride, a compound having local anesthetic action, and abolishing flexor spasm activity in intravenous dosage of 1 to 2 mg. per kilogram of body weight. Oral dosage sufficient to diminish spasm activity is apparently difficult to achieve below the level of toxicity, as was the case during this trial. Supplies of P–267, as well as two of the specimens described in Part III, were provided through the excellent cooperation of Carl A. Bunde, research director for Pitman-Moore Company and his associates, Benedict E. Abreu and George K. Burch. Their studies on the tremor syndrome and P–267 were reported in Fed. Proc., *11*, (1952): 20, 317, and 385.

plementary etherization permitted surgery.* By means of longitudinal skin incision and laminectomy of the twelfth thoracic vertebra, the dura was exposed. This phase of the procedure occupied one and a quarter hours, with much time spent in removal of the thick dense bone of the spine and laminae. Although an attempt was made to keep etherization minimal, hyperkinesia disappeared and was absent even when corneal and conchal reflexes could be elicited. Between 3:30 p.m. and 4:30 p.m., no supplementary ether was given, but it was possible to incise the dura and expose the subadjacent spinal cord. About 3:45 p.m., the hyperkinesia returned slightly in the left hindpaw, with a frequency of about 26 per minute. When it was thought that sufficient activity had returned to be definitive, solidified carbon dioxide, in chunks of pinhead to green-pea size, and in flakes, was packed closely and directly upon and about the exposed spinal cord. During this procedure, the hyperkinesia disappeared. Freezing was allowed for two minutes and at 4:37 p.m. the frozen cord was sectioned by coronal incision, the scalpel meeting woodlike resistance throughout, similar to that of balsa wood on cutting; no bleeding occurred. Although hyperkinesia remained absent, it was possible to elicit a patellar reflex almost immediately. Blood from the heart was withdrawn for special immunological studies, and necropsy was immediately performed. No gross abnormalities were apparent, and the entire brain and spinal cord were removed and placed in 10 per cent formalin.

TABLE 3—Effect of Oral P-267 on Spasm Frequency in a Tiger

Date	Time	Dose (mg.)	Effect on spasm frequency
May 7	8:30 a.m.	1,300	
	3:30 p.m.		40 per minute
8	11:00 a.m.		36 per minute, more forceful
	2:10 p.m.		36 per minute
	3:00 p.m.	1,400	38 per minute
9	9:35 a.m.	2,100	42 per minute, but forcefulness much less
	10:30 a.m.	.....	40 per minute, somewhat more forceful
	2:40 p.m.		Convulsion lasting 5–7 minutes
	5:15 p.m.	.....	64 per minute
	6:05 p.m.	.....	55 per minute
10	5:15 p.m.		32 per minute

Histological Study

Method.—Slabs 1 cm. thick were cut by coronal section through the anterior and middle cerebrum, and through the midpart of the cerebellum, and a slab 3 mm. thick was cut from one end of each thick slab. The thick slabs were embedded in celloidin, cut in serial sections at 30 μ, and every tenth, eleventh, and twelfth section stained by the methods of Mahon, Nissl, and hematoxylin-eosin, respectively. The thin slabs were embedded in paraffin, and cut in serial sections at 10 μ.

Blocks were cut from three segments of cervical cord, and these, with blocks of the thoracic and lumbar cord in serial sequence, were prepared for study by the method described for the cerebrum, the thin slabs being cut from the rostral end of each block.

Stained sections were arranged in rostrocaudal sequence, levels identified, and the histological appearance examined with ocular, low-power and high-power microscopic magnification.

Observations.—Right and Left Cerebrum.—Abnormalities were observed in the tissues in the region of the anterior and posterior sigmoid gyri, apparent to the naked eye in stained tissue. There were a few collections of lymphocytes within the cortical layers on both sides, occasionally observed to be organized about blood vessels. On the right in the rostral sections, there was a solitary small softening. On this side, also, there was a layer of gliotic tissue, approximately 1 mm. in thickness and several millimeters in width, running in a rostrocaudal direction and extending beneath the pia. Beneath this layer the structure of the cortex was disrupted; cells had disappeared, or appeared condensed by gliosis and shrinkage of the mesodermal tissues. The changes appeared to represent organized subpial hemorrhage and rare perivascular lymphocytosis with occasional small softenings.

Midcerebrum.—No abnormalities were observed.

Cerebellum.—No abnormalities were observed.

Spinal cord.—No abnormalities were ob-

* The authors acknowledge with thanks the assistance of the following individuals in this acute experiment: Berry Campbell, M.D., James Coder, Charles P. Gandal, D.V.M., and Mrs. Eugene Twigg, R.N.

served, although repeated visual comparative estimates were made between left and right sides of the cell columns and other structures. The concentration of neurons appeared similar and their morphology, and that of surrounding tissue cells and structure, showed no abnormalities. Several sample counts comparing incidence of cells in selected fields of anterior and lateral columns disclosed no significant differences between sides in lumbar and cervical regions. Specifically, no areas of demyelination or of perivascular lymphocytosis were observed.

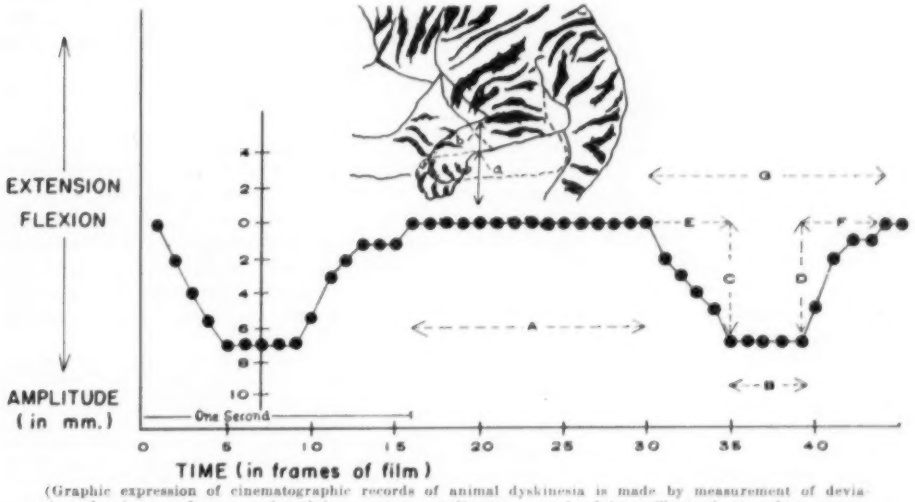
DISCUSSION

From the characteristics of the muscular activity and the effect of medications, there is no doubt that the specimen exhibited what can be conveniently designated as the flexor spasm syndrome. The flexor feature of the activity, invariable in our material, justifies its inclusion in the terminology suggested here. (*See* verbal description page 724 and graphic expression of activity, graph 1. As it will be further developed in Part III, definition of this syndrome is as follows: "Abnormal involuntary muscular activity identical in manifestations with the flexion reflex, but occurring without apparent stimulus, with movements composed in regular sequence and associated with paresis.")

The cause for the syndrome in this specimen is not known; indeed, the occurrence of the flexor spasm syndrome in dogs without prior clinical evidence of distemper or other disease complicated by neurological disorder is not uncommon. McGovern *et al.*[1] found 17 per cent of the animals they studied (as "tremor syndrome" specimens) had no clinical evidence of distemper.

The disorder has not previously been reported in the feline species, although "distemper," formerly referred to by some veterinarians, is now recognized to represent an enteritis associated with leukopenia and caused by a specific virus. It is a different virus from that causing distemper in the dog, which can infect ferrets and mink. A considerable degree of immunological difference is apparent, therefore, between carnivores of the families Canidae, Felidae, and Mustelidae.

It was interesting that no correlation



(Graphic expression of cinematographic records of animal dyskinesia is made by measurement of deviation of points on frames projected in sequence, as distance a to b of inset illustration, or by measurement of changes at the angles formed at joints of limb members, as illustrated by figure 1 of Part III. For details of this quantitative technique *see* J. R. Whittier, J. Comp. Neurol., *89*, [1948]: 1–19.)

Graph 1—Cinematographic record of spontaneous flexor spasm in a tiger. The animal is lying on the right side.

A and B—periods of maintained posture; C and D—amplitudes of flexor (contraction) and extensor (relaxation) movements, respectively; E and F—durations of flexor and extensor movements, respectively; G—duration of activity.

could be made between localization of dyskinesia maximum and neural pathological change, except for contralaterality; persistence below cord section rules out a limited cerebral lesion to explain the syndrome.

The opportunities for physiological and pharmacological research which the syndrome provides were demonstrated during the trials of treatment and medication. Tolerance for seconal varied in an inexplicable manner, although this medication permitted handling and instrumentation, in a state of light narcosis, of an otherwise dangerous animal. Unfortunately, the rauwolfia derivatives and chlorpromazine® were not available during these observations, or they could have been given as sedative adjuncts. The thorough inhibition of spasm activity by supplementary ether during the acute experiments was not anticipated and unfortunate. The effect of thiantoin in augmenting activity was remarkable and has been subsequently confirmed in other specimens. Data developed from this study are summarized in Part III of this series.

SUMMARY

1) An instance of flexor spasm syndrome in a tiger is described.

2) It was observed that the activity continued during sleep, was diminished by sedatives and abolished by ether, uninfluenced by a single period of artificial hyperpyrexia, markedly increased by thiantoin, and uninfluenced by tridione. A convulsion occurred during a single trial of oral P-267.

3) Transection of the spinal cord through a frozen segment was followed by no spinal shock effect on deep reflexes below the section. The effect of the transection upon the flexor spasm syndrome could not be determined.

4) Study of pathological changes of the neuraxis revealed abnormalities in cerebral tissue in the motor areas bilaterally, more marked on the right, as organized subpial hemorrhage, and perivascular lymphocytosis with small solitary softening. Specifically, no areas of demyelination were observed.

Reference

[1] McGovern, V. J., Wyke, B. D., Dodson, M. E., and Steel, J.: The Effect of Surgery upon the Canine Tremor-Rigidity Syndrome. M. J. Australia, *26*, (1948): 492–494.

III. Further Observations in Dogs

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Part I of this study reviewed the literature concerning the flexor spasm syndrome in carnivores, and Part II presented in detail a unique occurrence in a tiger. The observations to follow summarize experience and understanding in this laboratory to date.

MATERIAL AND METHODS

Between February, 1947, and June, 1953, 7 animals with hyperkinesia were provided which were suitable for study. Initial animals in the series, when submitted, were regarded only as interesting examples of naturally occurring hyperkinesia. Animals later in the series were subjected to systematic investigation, including (1) clinical observations and cinematography; (2) a variety of experimental procedures designed to confirm previously reported observations and to provide additional data; and (3) a study of pathological changes.* Material and methods are summarized in table 1.

RESULTS

The results of these studies are summarized in table 2 together with observations from other sources since Chauvau's[1] first report in 1862.

DISCUSSION

The flexor spasm syndrome can be recognized as a residual of canine distemper or of other neurological disorders.**

The syndrome may be associated with other motor symptoms following disease of the neuraxis, such as ataxia, ataxic tremor, and convulsions. The principal manifestations of the syndrome, when it occurs

The author acknowledges with thanks the assistance of Dorothy Logan and Marie Pappanicholas, and of Mae T. Seaman, secretaries of the Neurology Department and of Creedmoor Institute, respectively, and wishes to credit Gloria Oliver and Claudia Schogeloff of the Neurology Department for preparation of the histological material.

* The author's experience included a viewing of cinematographic records of the series of dyskinetic dogs demonstrating the inhibiting effect of P-267 medication on the flexor spasm syndrome, and presented by Carl A. Bunde on Feb. 6, 1952, to the Veterinary Medical Association of New York City, Inc., as well as study of a copy of the excellent color film provided through the courtesy of V. J. McGovern, Department of Morbid Anatomy, Royal Prince Alfred Hospital, Camperdown, Australia, demonstrating varieties of neurological disorder attending distemper in the dog.

** For consideration of the relation between distemper and the flexor spasm syndrome, *see* Part II, p. 723.

TABLE 1—Summary of Observations of Spontaneous Flexor Spasm Syndrome in Carnivores

Date	Case	History	Experimental procedures	Histological material	Pathological changes	Cinematographs
Feb. 1947	White terrier dog J528	Hyperkinesia in forelegs bilaterally without recognized distemper	Inconclusive results from neuroelectrical recording, Feb. 20 (acute experiment)	Cervical and thoracic cord cerebellar and cortical tissue	No significant changes	Obtained
Mar. 1947	Mongrel dog J571	Hyperkinesia in left hindleg preceded by racing fits	Regular bursts in peripheral nerve during neuroelectrical recording, Apr. 15 (acute experiment)	None	Not obtained	Not obtained
Apr. 1947	Black cocker dog J594	Hyperkinesia in abdominal wall preceded by distemper	Frequency increased (from 120/min. to 150/min.) after midsagittal section of medulla, level of corticospinal decussation; unexplained operative death occurred during attempted rubrospinal tractotomy	Marchi, of neuraxis tissue	Section as intended and degeneration in corticospinal tracts	Still photography only
1951–1952	Tiger "Seton" (*see* Part II)	Hyperkinesia in left hindleg primarily, without recognized distemper	Pharmacological effects of oral chloral hydrate seconal, thiantoin, P-267 Surgical effect of spinal cord freezing and section	Hematoxylin and eosin, Nissl, Mahon, of neuraxis	Old subpial hemorrhage and cerebral softening on right	Obtained
July 1952	Brown mongrel dog * P-M1	None available; hyperkinesia in right frontleg	Transection of cervical cord; surgical death	Hematoxylin and eosin, Nissl, Mahon, of cervicothoracic cord and dorsal root ganglia.	No significant changes	Obtained
Sept. 1952	White terrier dog ** "Nyack"	Hyperkinesia with paresis in both legs on left side; preceded several weeks by distemper	Pharmacological effects of intravenous picrotoxin and tridione, and surgical effects of spinal-cord freezing and section and of anterior-cerebral ablation (*see* table 2)	No material prepared	.....	Obtained
Dec. 1952	Brown mongrel dog * P-M2	None available; hyperkinesia in right hindleg	Pharmacological effects of oral quinine, intravenous P-267, and surgical effects of spinal cord freezing and section (*see* table 2)	Hematoxylin and eosin, Nissl, Mahon, of parts of cerebrum and stem, and entire cord.	Gliosis, ipsilateral lumbosacral segment	Obtained

* Provided by the courtesy of Dr. Carle Bunde, Pittman-Moore Company, Indianapolis 6, Ind.; ** provided by the courtesy of Morris Siegel, D.V.M., Nyack Animal Hospital, Nyack, N. Y.

TABLE 2—Summary of Results of Experimental Neuropharmacological Procedures on Spontaneous Flexor Spasm Syndrome, 1862-1953

Drug	Route	Effect on syndrome	Observer
Chloral hydrate	Rectal, oral	Decreased all characteristics	Le Gros, Onimus, 1870; Whittier, Goss, 1952
Ether	Inhaled	Decreased and abolished	Gowers, Sankey, 1877
Morphine	Parenteral	No change	Quincke, 1885
Barbiturates	Any route	Decreased and abolished	Many observers
Atropine	Parenteral	Increased all characteristics, and reversed quinine effect	Wood, 1893
Atropine	Parenteral	No effect	Abreu, personal communication
Picrotoxin	Parenteral	Increased all characteristics	Whittier, 1952
Tridione	Oral	No effect	Whittier, 1952
Thiantoin	Oral	Increased all characteristics	Whittier, 1952
Quinine	Oral, intravenous	Decreased and abolished	Wood, 1893; (not confirmed in this laboratory on one specimen)
P-267	Oral	Decreased and abolished	Bunde *et al.*, 1952
Artane	Oral	No effect	Burkhardt, personal communication
Artane	Oral	No effect	Abreu, personal communication
Mephanesin	Oral	No effect	Abreu, personal communication

unmixed with other symptoms, are abnormal involuntary activity combined with paresis or paralysis. The activity is composed of forceful contractions of affected muscle groups. It is of short duration, simultaneous wherever occurring, and involves only (in our material) flexor muscle groups in the limbs, the muscles of the abdominal wall, and nuchal musculature. Most commonly, as demonstrated by graphic analysis of motion picture films, there is a period of maintained posture, an interval during which no movement takes place. The movements appear, therefore, in a delayed sequence (graph 1, Part III, and graph 1, Part II) by which the activity can be distinguished from tremor. In 1 of the animals in McGovern's film, the movements occur in immediate sequence, and the activity appears as tremor. Unless, in such an animal, the activity is abolished by cord transection, which would indicate its dependence upon higher mechanisms, it is possible that the hyperkinetic component of the flexor spasm syndrome merges with the phenomenon of clonus (vide infra).

Characteristics of the flexor spasm syndrome between different animals vary in localization, frequency, amplitude, and forcefulness. In our material, it has appeared in both forelegs, one hindleg, both

TABLE 3—Summary of Results of Other Experimental Procedures on the Spontaneous Flexor Spasm Syndrome

Procedure	Effect	Observer
	NEUROSURGICAL	
Transection of spinal cord	Persisted below the section	Chauveau, 1862, *et seq.*
Transection of spinal cord	Persisted below the section, asynchronous with that above section, increased in amplitude, decreased in frequency	Wood, 1893
Section of dorsal roots	Decreased in characteristics	Le Gros, Onimus, 1870 Whittier, Carrea, 1952
Section of motor nerves	Abolished	Le Gros, Onimus, 1870 Whittier, Carrea, 1952
Section of corticospinal tracts	Persisted; increased in characteristics	Whittier, 1952
Ablation of anterior cerebrum bilaterally	Persisted	Whittier, 1952
Fracture of limb with shortening of involved musculature, muscle tenotomy, ventral and dorsal rhizotomy	Abolished	Turbes, 1954
	NEUROELECTRICAL	
Stimulation of spinal cord from above	Decreased amplitude	Le Gros, Onimus, 1870
Stimulation of spinal cord from below	Increased amplitude	Le Gros, Onimus, 1870
Stimulation of sciatic nerve	Persisted above tetany	Le Gros, Onimus, 1870
Stimulation of cerebral cortex	Increased frequency and amplitude	Horsley, 1886 Turbes, 1954
	MISCELLANEOUS	
Generalized pyrexia	Decreased characteristics	Traeger, 1948

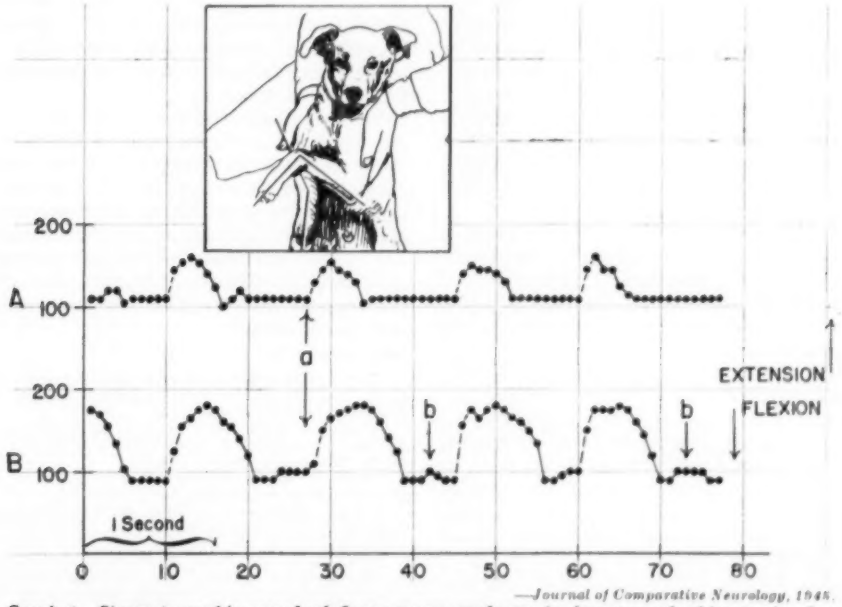
legs on one side, and in the muscles of the abdominal wall. (McGovern's film shows activity involving extraocular muscles.) Frequency in our material has differed between animals, from 40 per minute to 120 per minute; forcefulness, although varying between animals, has been difficult to measure. In the same animal, all characteristics are remarkably stable. Presence during sleep was invariable. A slight increase in frequency during the course of the day was usual, and a moderate increase when the animal was standing, in motion, or when in a state of alertness. Movements of forepaws and hindpaws are of dorsiflexion, confirming participation by this movement in the flexor withdrawal response as described by Walshe,[2] analogous to the Babinski toe sign in man.

It is the remarkable limitation of activity to the flexor muscle groups of the limbs, together with the persistence following transection of the spinal cord, that indicates a solution to the problem of terminology which has vexed understanding of this disorder (*see* Part I). The movements are the result of contractions of the flexor muscle groups, or flexor spasms, and in all respects but two are identical with the flexor spasms occurring after transection of the spinal cord in vertebrates.†

Walshe [2] described such a complete flexion reflex to consist of "a single flexion at hip and knee with dorsiflexion of foot and toes." He also observed that the extensors of the foot and toes in (or of the paw in a carnivore) participated physiologically as flexors, and enumerated the same muscles taking part in the flexion reflex as did Sherrington before him for animals (1910) and Kuhn [3] after him for man (iliopsoas, rectus femoris, sartorius, gracilis, pectineus, adductors longus and brevis, tensor fasciae femoris, semitendinosus, semimembranosus, biceps femoris, tibialis anticus, extensor longus digitorum, and extensor longus hallucis).

Kuhn [3] described the flexor spasm resulting from moderate plantar pin prick stimulus as follows: "The composite reflex action was then an abrupt, jerking, triple flexion, followed frequently by an

† Acknowledgment is due to Ernst Herz, M.D., Neurological Institute, New York City, for observing the similarity between the flexor spasm syndrome and the "mass reflex" of paraplegic human beings.



—*Journal of Comparative Neurology, 1948.*

Graph 1—Cinematographic record of flexor spasm syndrome in forepaws of white terrier dog J528, joint-angle measurement. Movements of dorsiflexion (extension) of paws are part of flexor withdrawal response.

equally abrupt relaxation of the entire limb. The reflex response thus resembled closely a muscle twitch in the steepness of the contraction and relaxation phases." Graphic expression of this activity as manifested in the flexor spasm syndrome is shown (graph 1, Part II, and graph 1, Part III).

The two differences between the flexor reflex and the spasms of this syndrome are (1) that the occurrence of the spasm does not depend upon an applied or eliciting stimulus, *i.e.*, they appear spontaneously, and (2) that they have regularity. The delicacy of the adequate stimulus for flexor spasms has long been known. Walshe [2] stated that "Whether these flexor spasms are actually spontaneous or not is uncertain. It seems probable that we are only dealing with a condition in which the threshold of stimulation is extremely low." It seems likely, therefore, that the spontaneous flexor spasm syndrome is a manifestation of the flexion reflex which depends for its inception and regularity upon a sequence of correlated changes in excitability in the neurons responsible for flexor activity.

Further evidence supporting this hypothesis consists of: (1) the coexistence of paresis and atrophy in affected muscles in all the specimens observed, suggesting interference with function of the spinal cord *per se*, (2) the autonomy of the activity within the spinal segment, already mentioned, (3) the presence during sleep, (4) the reported diminution following pyrexia,[4] observed for similar spasms in man by Kuhn [3] and others.

It seems probable that the syndrome is a specific stage in the sequence of residuals attending spinal cord disease in carnivores, often following distemper, which is to be compared with the third stage in the sequence enumerated by Kuhn and Macht [5] for man after severance of the cord. This syndrome includes: (1) spinal shock, (2) minimal reflex activity, (3) flexor spasms, (4) alternating flexor and extensor spasms, and (5) predominant extensor spasms.

It was not within the scope of this study to pursue, with further research, the leads which became apparent. The effect of quinine on the syndrome, in view of Wood's [6] report of 1893, has not received sufficient trial. The remarkable effect of thiantoin requires explanation, and it may be that the thienyl group of this congeneric substituted hydantoin is responsible for the marked increase in frequency and forcefulness which is so consistent, and so in contrast to the effect of other anticonvulsants. It is apparent that carnivores exhibiting this syndrome provide convenient specimens for physiological investigations of excitability of the central nervous system. Finally, it seems apparent that transection of the spinal cord by freezing may be followed by little if any shock below the level of the section, even though mechanical transection through the frozen region is subsequently performed.

SUMMARY AND CONCLUSIONS

1) Observations on the spontaneous flexor spasm syndrome in carnivores are presented.

2) The syndrome, unmixed with other symptoms, appears as abnormal involuntary activity combined with paresis or paralysis. The activity of affected muscle groups is composed of forceful contractions which are of short duration, simultaneous wherever occurring, and involve flexor muscle groups in the limbs. This activity persists during sleep, and persists in caudal segments despite transection of the spinal cord. It is diminished in characteristics by sedatives, which abolish it in sufficient dosage, and markedly increased in frequency and forcefulness by thiantoin medication.

3) The flexor spasms of the syndrome are identical with activity of the flexion reflex following transection of the spinal cord, except that they do not depend for their appearance upon an eliciting stimulus, and they appear in regular sequence.

4) The syndrome provides manifold opportunities for physiological and pharmacological investigation of the central nervous system.

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The Development of Palatability Tests for Cats

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THE PRACTICAL importance of providing rations which are palatable is unquestioned, but the factors which influence food acceptance are not well known. The work of Richter [1] and others [2-6] has shown that the choice of food may be determined by definite physiological need. This does not mean that habit, consistency, flavor, and various factors, other than the nutrient composition of the ration, are unimportant in determining whether or not one food is preferred to another. Young [7] concludes that "an animal accepts what he likes as well as what he needs and it is an open question as to how far what he likes agrees with what he needs."

Most of the studies upon food selection or appetite have been done with the laboratory rat. Although this animal has many obviously desirable characteristics for such studies and its nutritional requirements are well known, it is also relatively unfastidious and easy to feed. Dogs and cats are more likely to refuse rations or go off feed for unknown reasons. Studies in this laboratory have demonstrated that the cat is particularly fussy, although most healthy cats will consume purified rations composed of sugar, fat, casein, and the necessary vitamins and minerals.

Although the palatability of cat and dog rations is of considerable importance in their commercial production, relatively little has been written on this subject. This is particularly true for cats and, for this species, the study of nutritional needs is also fragmentary. During studies on cat nutrition, it became desirable to study the relative palatability or acceptability of different rations. Our experience in this field is described.

EXPERIMENTAL DATA

In the system finally devised, 15 adult cats were used for each test. These animals were housed in individual cages approximately 22 in. square and 16 in. high, equipped with ½-in. wire mesh bottoms. The animals were housed in an air-conditioned room for a considerable period and were accustomed to their surroundings and the laboratory personnel. Only cats consuming sufficient food to maintain their usual weight were used for the tests. When two food samples were compared, the animals were offered sufficient of each food in separate crocks to provide their total daily need. The following morning, the food remaining was weighed to determine the amount eaten. The crocks were washed, fresh food provided, and the position of the crocks in the cage reversed. Fresh water was provided *ad libitum*. In most routine tests we used two consecutive days, thus providing 30 comparisons when 15 animals were used.

In order to provide some information upon the effect of previous feeding on the choice of food and to partially eliminate it as a major variable, the animals when not on test were separated into three groups which received different commercial rations designated as X, K, and P. Five animals from each of these groups were used in each test. Two of these rations (X and P) were composed largely of fish and cereal, one of which was known to be more palatable than the other by previous tests. The third ration (K) was a commercial dog food of meat and cereal. All three were canned products.

RESULTS AND DISCUSSION

Statistical Evaluation.—The usual procedures for determining the difference between the two mean values [8] often failed to indicate statistical significance when the difference in intake between two foods was large, and it is apparent that this type of analysis is not appropriate for these kind of data. When an animal eats its usual quota of food, the amount of food A eaten must influence the amount of food B taken. In other words, the intake of foods A and B will be negatively correlated to a considerable degree and the variances are not independent of each other.

Several other types of statistical analyses were investigated. Considering the protocol of an experiment (table 1), one might determine whether the mean difference of 116 Gm. is different from zero by the usual "t" test.[9] While this difference is highly significant in the test shown, a consideration of the distribution of the values as discussed below may suggest that this approach is not entirely valid.

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The method of rank analysis as presented by Wilcoxon[10] and others[11] has also been used. In this method, the values in the difference column are ranked from 1 to 30 according to size, independent of sign. The sum of the plus and minus ranks, whichever is smaller, is then determined and evaluated according to Wilcoxon.[10] In a test involving 30 differences, a sum of 137 is significant at the 5 per cent probability level, 107 at the 1 per cent level. This method is simple for small numbers. It becomes very tedious for numbers larger than 25 or 30, although it can be applied. In most of the studies described, this test has been used to determine the significance of the results.

A much simpler test based upon the number of plus and minus signs in the difference column appears to be satisfactory in most instances. If there was no preference for either food, the number of plus and minus signs should be equal. In the actual test shown (table 1), there are only five negative signs and the probability that this would occur due to chance is much less than 0.01 by the chi-square test.[12] For the size of samples used here, where 30 differences are obtained, the chi-square test is significant at the 5 per cent probability level when there are nine values of one sign and 21 of the other.* If only seven are of one sign, the probability reaches 0.01. In a series of 31 tests, there were only two in which rank analysis indicated a significant difference which was not confirmed by the *chi*-square test. Since the *chi*-square test is so easily applied, particularly if the numbers are increased, this may be the method of choice for routine tests.

TABLE 1—Protocol of a Test Comparing Rations A and B

Cat*	Day	Food eaten A (Gm.)	Food eaten B (Gm.)	Difference (Gm.)	Rank**
X–1	1	210	100	110	12
	2	200	80	120	13
2	1	60	90	−30	−2.5
	2	150	80	70	8
3	1	80	85	−5	−1
	2	80	50	30	2.5
4	1	200	100	100	11
	2	130	80	50	4
5	1	130	60	70	8
	2	230	45	185	18.5
K–1	1	320	40	280	29
	2	200	30	170	16
2	1	280	40	240	25
	2	250	25	225	24
3	1	230	35	195	20
	2	220	35	185	18.5
4	1	230	30	200	21.5
	2	220	40	180	17
5	1	290	40	250	26
	2	180	30	150	14
P–1	1	40	430	−390	−30
	2	150	240	−90	−10
2	1	340	80	260	27
	2	190	30	160	15
3	1	230	30	200	21.5
	2	75	20	55	5
4	1	320	250	70	8
	2	230	290	−60	−6
5	1	420	150	270	28
	2	350	130	220	23
	Mean	208	92	116	
	Sum of minus ranks				−49.5†
					$p < 0.1$

* The cats used received either ration X, K, or P when not on test as indicated; ** *see* text for explanation; † evaluated according to Wilcoxon.[10]

Pattern of Food Selection.—It might be assumed that if the same kind of food were placed in the two dishes so that the animals presumably had no basis for making a choice, each animal would consume a similar amount from each dish. This has been tested, using 15 cats for two days as described previously. For the entire group, the average intake was 120 Gm. from the dishes arbitrarily designated as A and 131 Gm. from the dishes designated as B. This small difference would suggest that, as expected, the animals did not make a choice since the same kind of food was supplied in each dish. However, if the animals eat randomly from either dish, one would expect the amounts of food eaten from either dish to be distributed according to a "normal" curve around the mean intake of about 125 Gm. Most of the time, values approaching 125 Gm. should disappear from the dishes and only infrequently should very small or large amounts disappear from a dish. From the data available in the test, the number of times that food selections of different amounts should occur can be calculated,[13] assuming that the selections were made entirely by chance (graph 1).

It is readily apparent that the data are not normally distributed. There is a preponderance of small and large food selections and relatively few between 120 and 180 Gm. This means that when the same food is offered in each dish, the animals do not eat randomly from either dish. Rather, they tend to consume most of their food from one dish or the other. Why this should be true is not clear, but it has been confirmed in a large series of trials in

* As many as 16 of the 30 values could be of one sign and yield a significant difference by rank analysis.

which the foods were so similar that no choice could be made. Since most of the common statistical tests, such as the "t" test, are based upon the assumption that the data are normally distributed, their application may be criticized when applied to data where this condition is not met.

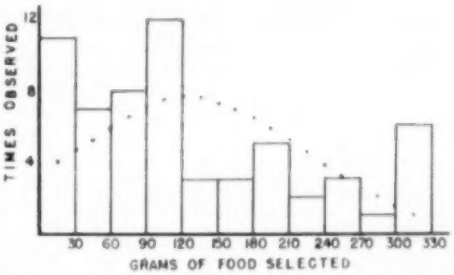
Position of Food Dishes.—As indicated, the position of the food crocks was reversed in the cage each day. It is possible that some animals may prefer a particular side of the cage or "eat according to position." In order to test this possibility, we have analyzed some of the data available from tests, in which no preference was demonstrated, according to position in the cage rather than the food sample. We have been unable to show that the position of the feeding crock is a determining factor in the choice of food.

Preference of Individual Animals.—The problem arises as to whether there are differences in cats; that is, whether certain animals consistently prefer a certain type of food and are different from the average group of cats. To test this possibility, we had available 15 animals which had been used in four separate tests in which a particular food (ration P) was one of the choices. In each of these tests, ration P was preferred to a statistically significant extent by the entire group of animals over the other ration tested. The combined data for each cat were tested by rank analysis (table 2). Three of the animals showed a

TABLE 2—Food Preferences in Four Tests Where Ration P Was One of the Choices

Cat	Preference	Cat	Preference	Cat	Preference
X–1	Other	K–1	P	P–1	Other
X–2	P	K–2	P	P–2	P
X–3	P	K–3	P	P–3	P
X–4	P	K–4	P	P–4	P
X–5	No choice	K–5	Other	P–5	P

statistically significant preference for the ration fed other than P, 1 animal made no signficant choice, and the remaining animals preferred ration P. There appears to be little doubt that some cats show food preferences different from the average. This finding, of course, imposes some serious questions upon palatability tests, since the results might be inadvertently determined by choice of animals used. Presumably, the use of sufficiently large



Graph 1—The bars represent the number of times that food selections of different amounts were observed from either of two food dishes when the same food was offered in each dish. The dotted line represents the theoretical distribution which the data should follow if the choices were made entirely by chance. It is apparent that the data do not fit this expected "normal" curve.

groups of animals will minimize the effect.

Effect of Previous Rations.—Another question which we attempted to investigate was whether the previous ration influenced the food choice. It will be seen (table 2) that, in the tests presented, there was no indication that the animals accustomed to rations X, K, or P responded differently. This might, however, depend upon relative difference in acceptability between the rations studied. When the differences are small, for example, and the animal faces a more difficult discrimination, factors may become important which are not apparent when one of the rations is greatly preferred to the other. Three groups of 10 animals each were used to study the effect of the previous ration. These groups had received either ration X, K, or P prior to the test. During the first week, the food intake while feeding these rations was determined. The cats were then separated into groups of 5 which were allowed certain choices (table 3). For the first four test weeks, one of the choices was always the ration to which they were accustomed. During the seventh week, they were allowed a choice between the two of the three rations to which they were not accustomed. Other details of the study are apparent from table 3.

In the first four groups, A to D, which had previously been fed either ration X or K, the animals with one exception chose ration P whenever this was offered. The single exception was the third week of group B, and here too the intake of P was somewhat higher than of X, although not

TABLE 3—Effect of Previous Ration Upon Choice and Amount of Food Consumed

Group*		Week of test							
		1	2	3	4	5	6	7	8
A	Food offered	X	X P	X K	X P	X K	X	P K	X
	Total eaten (Gm.)	209	286	255	266	260	200	303	180
	Choice (Gm.)		87 199	146 109	95 171	129 131		176 127	
	p		.01	N.S.	.02	N.S.		.01	
B	Food offered	X	X K	X P	X K	X P	X	P K	X
	Total eaten (Gm.)	192	266	248	219	236	146	204	175
	Choice (Gm.)		135 131	114 134	112 107	90 146		126 78	
	p		N.S.	N.S.	N.S.	.05		.01	
C	Food offered	K	K X	K P	K X	K P	K	P X	K
	Total eaten (Gm.)	150	227	227	205	198	130	232	167
	Choice (Gm.)		70 157	53 174	73 132	55 143		161 71	
	p		.01	.01	.01	.01		.01	
D	Food offered	K	K P	K X	K P	K X	K	P X	K
	Total eaten (Gm.)	156	259	218	223	221	227	291	174
	Choice (Gm.)		55 204	65 153	52 171	99 122		197 94	
	p		.01	.01	.01	N.S.		.01	
E	Food offered	P	P X	P K	P X	P K	P	K X	P
	Total eaten (Gm.)	173	161	290	229	252	134	251	186
	Choice (Gm.)		81 80	48 242	131 98	121 131		198 53	
	p		N.S.	.01	N.S.	N.S.		.01	
F	Food offered	P	P K	P X	P K	P X	P	K X	P
	Total eaten (Gm.)	200	165	298	283	268	153	266	239
	Choice (Gm.)		63 102	84 214	160 123	162 106		185 81	
	p		.01	.01	N.S.	.05		.01	

* Groups A and B accustomed to ration X; C and D to ration K; and E and F to ration P; p—probability that results are due to chance as determined by rank analysis; N.S.—not statistically significant.

to a significant degree. On the other hand, in groups E and F which had previously been accustomed to ration P, there was only one test (group F, week 5) where ration P was preferred. In the other seven trials involving ration P, the animals either did not make a significant choice or preferred the ration other than P. Similarly, it may be seen that in none of the tests with groups A and B was ration X preferred, and these animals consistently failed to distinguish between rations X and K. On the other hand, animals accustomed to ration K (groups C and D) preferred X over K in three of the four comparisons made, and in none of the trials was ration K preferred. It would appear that in these trials, at least, there was a general tendency for the animals to prefer the ration to which they were not accustomed. This is an unexpected finding, perhaps, and must be considered important in the setup of palatability trials, since the results may be severely biased by the previous ration the animals received. Considerably more work is required to determine the influence of this effect and also the means to eliminate or minimize it. While we have routinely used animals accustomed to three different rations, there is no assurance that this is satisfactory. Other approaches might be to feed a group of different rations at random to the animals so they are not accustomed to any particular one. It may also be suggested that this effect warrants consideration in field trials with consumers. The results obtained might be influenced by the predominant cat food in use in an area and how similar the product being tested is to the predominantly used food.

It would appear also that the total food intake was generally higher when a choice of foods was offered (table 3). During weeks 1, 6, and 8, the mean consumption was somewhat less than during the other weeks. This may also suggest that the animals prefer a new food.

Length of Test.—In view of the observation that the previous ration may influence the choice, a question arises as to the value of a short test or how a short test may compare with a longer one. The results of a test upon 15 animals, where the food choices were followed for six days, are shown in table 4. In the analysis, the first three days of the test were compared with the second three days. Whereas in the first three days no significant difference in rations A and B under test could be shown, ration B was preferred to a significant extent during the second three-day period. This was due mainly to a decreased intake of ration A by the animals accustomed to ration K. The observation does raise questions, of course, as to the value of short tests. We feel that if a ration appears unsatisfactory in a short test,

TABLE 4—The Effect of the Length of the Test Upon Food Choice

Period	Cats*	Food consumed, Gm. Ration A Gm.	Ration B Gm.	Difference Gm.	p
First 3 days	X	174	143	31	
	K	109	166	−57	
	P	166	175	−9	
	Mean	150	161	−11	N.S.
Second 3 days	X	133	120	13	
	K	48	165	−117	
	P	147	133	14	
	Mean	109	139	−30	0.02

* Five cats in each group accustomed to rations X, K, and P as indicated; N.S.—not significant at 5% probability level.

it is unlikely to prove satisfactory under more extensive trial. The short test may thus be a valuable guide, but final acceptability of a ration will obviously require more extensive testing. It is possible that change in acceptability might reflect a nutritional inadequacy which only becomes apparent with time. In view of the little knowledge of the factors which influence food acceptance, it is unlikely that screening methods will serve to classify various rations in all aspects. We have observed rations which had a high acceptability by the test described but which were nutritionally inadequate to support health in the animals over an extended period. A ration can not be considered satisfactory unless it is acceptable to the animals, but the fact that it is acceptable does not necessarily mean that it is satisfactory.

CONCLUSIONS

Attempts to devise a reasonably satisfactory screening test for the palatability of rations for cats, which is rapid and sufficiently sensitive for the purposes desired, have been described. A test utilizing 15 adult cats over a two-day period was sufficient to differentiate between two rations in many of the tests. The sensitivity of the test can presumably be improved by increasing the number of cats or the number of days of the test.

The data have been analyzed by rank analysis. In most tests, a simple *chi*-square analysis based upon the number of times one ration is preferred over another appears adequate to evaluate the results.

Some animals had definite food preferences which were different from the majority; thus, the selection of the animals used may be crucial. In general, there was a tendency for the animals to choose a new ration rather than the one which they were usually fed. Also, when the animals were offered two foods over a period of time, their preference sometimes changed significantly. While we are unable to offer means whereby these limitations may be eliminated, the investigator should be familiar with their occurrence.

The fact that animals prefer a ration does not necessarily mean that it is satisfactory. Certain foods of high acceptability were not nutritionally adequate over an extended period. Conversely, foods which appear nutritionally adequate may have a low acceptability when offered in the presence of other rations.

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Further Studies on the Evaluation of Immunity in Chickens Vaccinated with Formalin-Inactivated Newcastle Disease Virus Vaccine

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THE METHOD most commonly employed in evaluating immunity response following use of Newcastle disease virus (NDV) vaccine is to administer the challenge virus intramuscularly and to interpret the results on the basis of survival of the vaccinated chickens.

Brandly *et al.*[1] have studied in some detail means of evaluating the immunity response to NDV vaccines. Comparative studies made between routes of challenge exposure indicatad that the most severe test of refractivity was the intravenous route, followed in order by intramuscular and oral exposure. Evaluation of immunity was on the basis of mortality, length of survival, and signs of illness. Taylor[2] is of the opinion that the challenge dose should be given by the normal route of infection (intranasal) and that mortality should be the only criterion in measuring immunity in young chicks, while in adult birds both mortality and interruption of egg production should be considered. Zuijdam[3] recovered virus from the feces following challenge oral exposure of chickens that had been vaccinated with formalin-inactivated NDV vaccine, even though up to 80 per cent of the birds did not show clinical signs of illness.

The author reported on a method of evaluating immunity on the basis of recovery of the virus from the blood and tissues 96 hours after challenge exposure by the intranasal route. Johnson *et al.*[5] have emphasized the importance of better methods of testing the potency of vaccines, particularly as to the degree and duration of immunity. These authors suggested a potency test similar to the one employed for the testing of some other vaccines in which the challenge virus is titrated in both the control and the vaccinated chickens, the difference in titer representing an index of the degree of immunity.

The purpose of this investigation was to evaluate the immunity in a group of chickens given two doses of inactivated vaccine, employing the potency test as suggested by Johnson *et al.*[5] Both survival of the bird and virus recovery from the blood on the fourth day after challenge were used as criteria for determining resistance to challenge.

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PROCEDURE

Two hundred and eight New Hampshire crossbred chickens were injected intramuscularly with a commercial, formalin-inactivated, alumina-gel adsorbed NDV vaccine using two doses; an initial 1-ml. dose was injected at 6 weeks of age and a second 1-ml. dose at 22 weeks of age. Birds of both sexes were included, and 60 control chickens were kept in direct contact with the vaccinated birds during the experiment. Immunity of the birds was challenged with the GB-Texas-1948 strain of NDV two to three months after the second dose of vaccine. Just prior to challenge, blood samples were taken from 30 chickens. Antibody titers were ascertained by the serum-neutralization (SN) and hemagglutination-inhibition (HI) procedures. The HI *beta* test was employed using 10 hemagglutinating units of formalin-inactivated virus in each of a series of twofold dilutions of serum. The serum dilutions were prepared directly in the 0.5-ml. virus suspensions. Washed chicken red blood cells were used at a concentration of 0.25 per cent in 0.5-ml. amounts to detect specific inhibition. Strain 174-Iowa-1947 was used as antigen.

The vaccinated and the control birds were separated for challenge into three groups of approximately equal numbers of males and females, with 60 vaccinated and 20 control birds in each group. An attempt was made to determine the immunity of the 60 vaccinated chickens of group 1 by inoculating dilutions of GB virus-infected allantoic fluid intranasally, 20 chickens for each dilution (undiluted, 10^1 and 10^2). The dosage was 0.1 ml. (0.05 into each nostril). The virus was also titrated simultaneously in the unvaccinated controls, using 10^4, 10^5, 10^6, and 10^7 dilutions of infected allantoic fluid. The infective titer for chickens was determined by demonstration of virus in the blood at 96 hours after challenge. Heparinized blood samples were collected from each bird by heart puncture. The blood samples were refrigerated until the next day, centrifuged, and the plasma decanted and replaced with tryptose phosphate broth (pH 7.3). The resuspended cells, in 0.2-ml. doses, were then inoculated into 11-day-old embryonating chicken eggs. Following collection of blood samples at 96 hours, the controls were destroyed but the vaccinated birds were observed for an additional ten days.

TABLE 1—Immunity Challenge with GB-Texas-1948 Strain of Newcastle Disease Virus by Three Routes of Inoculation Among Chickens Vaccinated Twice with Newcastle Disease Formalin-Inactivated, Alumina-Gel Adsorbed Vaccine

Route of challenge	Dilution of challenge virus	No. challenged	Chickens resisting infection*		Chicken-infective titer of virus in		Serum testing** log of geometric mean	
			(No.)	(%)	Controls	Vaccinated	SN test	HI test
Intranasal	10^2	20	19	95	$10^{6.0}$	$<10^1$	5.9	3.5
	10^1	20	14	70				
	Undil.	20	10	50				
Intramuscular	10^3	20	20	100	$10^{3.5}$	....	6.0	3.4
	10^2	20	20	100				
	10^1	20	20	100				
Aerosol†	10^1	20	10	50	$10^{5.4}$	$<10^1$	6.4	3.3
	Undil.	20	10	50				
	Undil. x 2‡	19	9	47.3				

* No virus recovery from blood; ** based on 10 samples from each group; † 110 strokes per cage with a hand-pumped nebulizer; ‡ 220 strokes per cage.

A chicken was considered resistant to challenge if virus was not recovered from the blood and if the bird survived without signs of Newcastle disease.

Determination of immunity in the 60 vaccinated chickens of group 2 was attempted by intramuscular inoculation of a 0.1-ml. dose of GB virus-infected allantoic fluid at dilutions of 10^1, 10^2, and 10^3. Twenty chickens were used for each dilution. The virus was also titrated in the unvaccinated controls, using 10^6, 10^7, 10^8, and 10^9 dilutions of infected allantoic fluid. To detect the presence of infection in group 2, the same technique as applied in the intranasal challenge of group 1 was used.

The immunity of the third group of vaccinated birds was challenged by an aerosol exposure of GB virus employing a hand-pumped peralta nebulizer.* The birds were placed in closed isolation cages and exposed continuously for ten minutes, the dosage being governed by the number of strokes employed in the operation of the nebulizer. Of the 60 vaccinated chickens, 20 received 110 strokes of a 10^1 dilution of infected allantoic fluid, 20 received 110 strokes of undiluted material, and the remaining 19 received 220 strokes of the undiluted infected allantoic fluid. After exposure, the birds were removed and placed in batteries in isolated rooms 10 ft. by 12 ft. which had separate ventilation. The virus was titrated in the unvaccinated controls using 10^4, 10^5, 10^6, and 10^7 dilutions of infected allantoic fluid by aerosol.

RESULTS

The procedure and results are presented in table 1. Following the intranasal inoculation of 0.1 ml. of undiluted infected allantoic fluid, 10 (50 %) of 20 chickens resisted the challenge. In the 10^1 dilution, 14 (70 %) of 20 chickens were refractory, and in the 10^2 dilution, 19 (95 %) of 20 were resistant. Thus, the chicken-infective titer of the challenge virus was approximately 10^1 in the vaccinated brids. A chicken-infective virus titer of $10^{6.0}$ was determined in titration of the virus in 20 unvaccinated controls.

Following challenge by the intramuscular route, virus could not be recovered from the blood at 96 hours, even though the birds received a greater amount of virus than did the birds in group 1. Titration of the virus in the unvaccinated controls indicated a $10^{3.5}$ titer.

Following the aerosol challenge with 10^1 dilution of infected allantoic fluid, 10 of 20 chickens resisted infection. After exposure to undiluted material, 10 of 20 chickens resisted infection. With doubling of the undiluted dosage (220 strokes), 9 of 19 resisted infection. Even though the degree of exposure was varied, approximately the same number of birds resisted the dilutions of virus. The virus-infective titer for the vaccinated birds was $<10^1$. The titer in the unvaccinated controls was $10^{5.4}$.

Distinct nervous or respiratory signs were absent among these chickens during the ten-day period following challenge, and no mortality occurred in any of the three groups. No critical evaluation could be made of the effect of challenge on feed consumption or egg production because the chickens were removed from their isolation quarters, where they were on litter, and placed in wire-floored batteries.

Tests of ten serum samples from each group taken prior to challenge revealed a fairly substantial antibody titer. The logarithm of the geometric mean SN antibody titer was 5.9, 6.0, and 6.4, respectively, in the three groups of vaccinated birds. The corresponding mean HI antibody index was 3.5, 3.4, and 3.3 (approximately a

* Obtained from Peralta Hospital, Oakland, Calif.

1:320 dilution of serum inhibiting 10 HA units of virus) in the three groups.

DISCUSSION

While not included in table 1, results of immunity challenge of groups 1 and 3 indicated the males to be more resistant than the females, e.g., in group 1, 12 females and 5 males were susceptible, and in group 3, 22 females and 8 males were susceptible. The 88 vaccinated males and 91 vaccinated females were placed in three groups at the time of challenge with the sexes represented as equally as possible.

While it was not possible to detect tenfold differences in size of challenge dose when results were based on signs of illness or mortality following challenge, this difference was apparent when evaluation was based on virus isolation from the blood, as revealed by the challenge results obtained from the vaccinated chickens in group 1. These results are significant and are in agreement with those of Zuijdam[3] who found that vaccinated birds may have sufficient immunity to prevent signs of infection and mortality but, nevertheless, were susceptible to challenge and excreted the virus. Johnson *et al.*[5] pointed out the importance of measuring the degree of immunity engendered by the vaccine in question in order to fully evaluate the product. Since the GB challenge virus causes a transient viremia, even in birds having high serum antibody titers, the removal of a blood sample to detect infection after challenge is relatively simple and the birds can be held for further observation. It appears that titration of the challenge virus in vaccinated chickens by the intranasal route is feasible and that the difference between titers in the control and the vaccinated birds might be a reliable index of the immunizing potency of an inactivated NDV vaccine.

In group 2, evaluation of the results of intramuscular challenge, on the basis of virus recovery from the blood at 96 hours, was not satisfactory in view of the results obtained in the group challenged intranasally. It is possible that blood samples collected earlier would have revealed differences between the doses of challenge virus used. Since the infective titer for the control birds was a hundredfold greater by the intramuscular route than by the intranasal route, the actual challenge was greater in the second group than in the other groups. Further work is needed on evaluating immunity following intramuscular challenge, as compared with the intranasal route, using virus recovery from the blood as the criterion of infection.

Determination of the chicken-infective titer of NDV in vaccinated chickens by means of aerosol exposure was not entirely satisfactory. It appears from this experiment that it will not be possible to detect tenfold variations in challenge inoculum by aerosol exposure as was done by the intranasal route. While an aerosol infection is accomplished chiefly by the intranasal route, there appears to be a difference, under the conditions of this experiment, between aerosol exposure and intranasal inoculation. An aerosol of the virus can be expected to result in an exposure of a larger number of cells in the respiratory system than following intranasal inoculation. Chickens exposed to an aerosol usually manifest respiratory râles earlier and more uniformly than chickens given an intranasal inoculation. This can be demonstrated with infectious bronchitis virus as well as with NDV. While it is difficult to determine dosage of aerosol exposure, it is estimated that the actual amount of virus the birds inhale is much less per bird than the 0.1 ml. inoculated intranasally.

SUMMARY

Measurement of immunity in chickens vaccinated twice with inactivated Newcastle disease virus (NDV) vaccine was attempted by employing a potency test in which the challenge virus is titrated in both the vaccinated and the control chickens. The difference in titers between the vaccinated and the control chickens represents an index of the degree of immunity. The intranasal, intramuscular, and aerosol methods of challenge were employed. Infection in the titrations was based on virus recovery from the blood at 96 hours following challenge. After this, the controls were destroyed and the vaccinated birds observed for an additional ten days. Following intranasal challenge, the chicken-infective titer of the virus in the vaccinated birds was approximately 10^2, while in the controls it was 10^6. Thus, the vaccinated birds were able to withstand 10^5 chicken-infective doses by the intranasal route. Following intramuscular challenge,

the virus-infective titer in the controls was $10^{6.5}$; however, in the vaccinated birds the titer could not be determined. The aerosol exposure resulted in a titer of $10^{5.4}$ in the controls, while in the vaccinated chickens the titer was estimated to be $<10^1$. None of the vaccinated chickens in the three groups became clinically ill or died in the ten-day observation period after challenge. Serum antibody testing revealed a substantial antibody titer by both the serum-neutralization and the hemagglutination-inhibition tests.

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A Diphtheroid Stomatitis of Chickens Apparently Due to a Spirillum, *Spirillum Pulli*, Species *Nova*

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ADULT CHICKENS of several breeds (Rhode Island Red, White Leghorn, and White Plymouth Rock) were observed to have diphtheritic lesions in the mouth. These were yellowish white, rather firm, and adherent to the underlying normal tissue. Quite often these lesions were symmetrical ovoids, one at each side of the lower jaw, about 0.5 inch distal to the commissure of the mouth (fig. 1). Lesions * were often found on the palate, on the lower surface of the tongue, on the floor of the mouth, between the larynx and the transverse row of papillae on the tongue (fig. 2), around the larynx, and on the walls of the pharynx. The size of the lesions varied from about 2 to 20 mm. in greatest dimension although most were about 8 mm. in greatest dimension.

PREDOMINANT ORGANISM

Dark field microscopic examination of wet smears of the lesions revealed the presence in large numbers of a spiral organism, along with other bacteria. The spiral organism had a rigid body with rounded ends and what appeared to be a single flagellum at either end (fig. 3). It usually had from three to six spirals and varied in length from 5 to 12 μ. It was about 1 μ in diameter The cytoplasm contained refractive granules almost as wide as the organism. The organism was actively motile, often darting back and forth so rapidly as to be almost invisible. The flagellum at the leading end was invisible while that at the trailing end was a blur, often making the organism look like a very narrow V. Sometimes, the organism would stop momentarily, enabling one to distinguish the spiral form and the flagella. In most wet smears, motility was lost rapidly. When allantoic fluid or serum were added as diluents, motility was retained for several hours.

Smears made from the lesions were fixed with methyl alcohol and stained with Giemsa stain. The spiral organism did not stain uniformly; there were round areas scattered along the body, of a diameter approximately that of the organism itself, and staining dark purple in contrast to the remainder of the cell which was light blue or gray. Flagella could not be seen and the appearance of the spirals was much flatter than that of the dark field preparations. The round areas, apparently the refractive granules seen in wet smears, varied in number (as many as 10 being seen) but were usually about one per coil.

Other smears of the lesions were fixed with heat and stained by the Gram method. The appearance of the spiral organisms stained in this way was difficult to classify. There were purplish areas, unstained areas, and pinkish areas in each cell. The pink areas seemed to be most outstanding. The unstained areas seemed to be about the same size and number as the dark purple areas seen with Giemsa staining.

Wet smears made from salivary glands of affected birds were found to contain large numbers of the same spiral organism. Smears made from the mouth and from the salivary glands of birds in flocks where no lesions were found did not contain the spiral organism.

CULTURE ATTEMPTS

Attempts were made to culture the organism on, or in, the following: tryptose blood agar, serum agar, tryptose agar with salivary gland infusion, veal infusion broth, veal infusion broth over tryptose blood agar slants, veal infusion broth with added salivary gland infusion, veal infusion broth with 10 per cent chicken serum, veal infusion broth with 10 per cent washed chicken erythrocytes, allantoic fluid, Stuart's medium, 9-day-old chicken

Portions of this work were done at the Poultry Division, University of Georgia Experiment Station, Athens, Ga., and at the Pitman-Moore Co., Zionsville, Ind. Acknowledgment is made by the author of the expert assistance of Mrs. Thomas Lewis.

* Similar lesions and apparently similar organisms were seen in chickens in the Mexico City area of Mexico, when the author was investigating poultry disease problems in the laboratories of the School of Veterinary Medicine of the University of Mexico, and in the laboratory of Industrias Modernas Alimentos Balanceados, S. A.

embryos (yolk sac, allantoic cavity, chorioallantoic membrane). In all of these mediums, the spiral organism died out rapidly, being overgrown by miscellaneous organisms. Some tubes and plates of the artificial mediums were incubated at room temperature, others at 30.0 C. and others at 37.6 C. The size of the inoculum varied from 0.1 cc. to 1.0 cc. In a few instances, serial tenfold dilutions of the inoculum were made up to a final dilution of 10^{-12}. Some of the culture attempts were made under an atmosphere of approximately 10 per cent CO_2, as well as under normal atmosphere. The mediums were checked for the presence of spirilla daily for a week after inoculation and then at irregular intervals for a month or, in a few instances, three months. Acid mediums killed the spirilla rapidly.

Nine-day chicken embryos were used in attempts to grow the spirilla. Some eggs were inoculated on the chorioallantoic membrane by the window method. Other eggs were inoculated in the allantoic sac by means of a 22-gauge needle inserted through the air sac end of the egg. Still other eggs were inoculated in the yolk sac by means of a 22-gauge needle inserted through the air sac end of the egg. Inoculated chicken embryos were then incubated in some instances at 39.0 C. and in other instances at 35.0 C. The size of the inoculum was usually about 0.25 cc. In one instance, serial dilutions of inoculum in allantoic fluid up to 10^{-14} were made and 12 eggs inoculated with each dilution. Eggs with embryos dying before hatching were examined for spirilla by dark field examination of the extraembryonic membranes, the extraembryonic fluids, the embryonic blood, and other embryonic tissues. Small (0.01 cc.) samples of allantoic fluid were drawn by means of Pasteur pipettes from eggs with living embryos at intervals during incubation and examined for spirilla. At hatching time, smears of the chick tissues were examined by dark field study.

Experimental Passage of the Disease

Lesions identical in appearance with those occurring naturally were produced by inoculating scrapings of characteristic lesions containing spiral organisms into the buccal mucous membrane of 6-week-old chickens. Inoculation with a 20-gauge needle was made through a film of mucosal scraping suspension by multiple shallow puncture of the mucous membrane. Lesions appeared in about ten days. Uninoculated birds, kept in the same cage with the inoculated birds, developed lesions in about 14 days, while uninoculated birds kept in other cages did not. Spiral organisms were found in the lesions of the inoculated birds and in the lesions of their cage mates. About six weeks postinoculation, large lesions were not usually seen although small lesions could be found here and there in

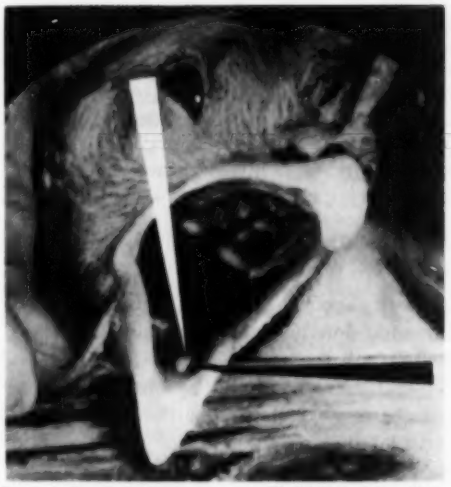

Fig. 1—Large lesion (arrows) of chicken stomatitis.

Fig. 2—Small lesions (arrows) of chicken stomatitis on the tongue.

the mouth and pharynx. Spiral organisms could be found in smears made from the small lesions and also in smears made from the salivary glands of inoculated birds and cage mates. The experimental birds apparently were not affected in general health by the infection, although weight records were not kept.

ATTEMPTS TO DEMONSTRATE FOWLPOX VIRUS

Sections of the lesions were examined for inclusion bodies of fowlpox but none was found.

Scrapings from the lesions were suspended in physiological saline solution and inoculated into the feather follicles and combs of 5 6-week-old chickens not vaccinated against fowlpox. No lesions were observed to be produced in two months.

EXAMINATION FOR FUNGI

Smears made from lesions were cleared with 10 per cent potassium hydroxide solution and examined microscopically. Fungous cells were not found.

Fig. 3—Dark field view, of wet smear of scraping from a mouth lesion of a chicken, showing Spirillum pulli. Organism had become inactive, allowing flagella to be seen. About x 3,000.

DISCUSSION

The lesions described above are apparently those of a heretofore undescribed minor disease of chickens. Grossly, the lesions resemble those of fowlpox but inclusion bodies were not found on microscopic examination, and fowlpox virus was not demonstrated by chicken inoculation.

Jasmin [1-3] found spirochetes in the respiratory tract of chickens and sparrows in Montana. He did not describe the spirochete found. "Marked lesions" were not observed on necropsy.[2] Stained smears sent to the author by Jasmin contain organisms resembling those seen in stained smears of the spirilla herein described.

Krage and Weisgerber[4] found two types of spirochetes in a respiratory disease of chickens. One of these was somewhat like the organism of the present report, since these authors reported that the organism resembled most (except for being about 3 times larger) the cause of rat-bite fever, *Spirillum minus*. On the other hand, Krage and Weisgerber stated that their organism was pointed at the ends; they made no mention of any dark round bodies in their description of the Giemsa-stained organism. The drawings accompanying their description, made from negatively stained preparations (Burri stain), resemble somewhat the organism of the present report. They claimed that a high percentage of healthy chickens contained the spirochetes in the pharynx, although in small numbers.

Gibbs,[5] in a study of microorganisms found in the respiratory tract of chickens, found spirochetes in 50 per cent of birds dying of "infectious trachitis," in 100 per cent of birds with chronic laryngitis, in 40 per cent of birds with avian paralysis, and in 30 per cent of healthy birds. Flagella were not observed in these spirochetes.

Harris[6] studied spirochetes found in sick chickens and in apparently healthy chickens in the Baltimore area and reported: "Examinations of the throat swabs seldom showed spirochetes, and when present they were coarse S-shaped with two or three loosely coiled spirals and pointed ends."

Carpano[7] reported a new genus and new species of spirilla, *Microspirillum avium*, from the blood of bullfinches *(Pyrrhulea europaea)* dead of disease in Egypt

shortly after importation from Berlin. The birds had shown ruffled plumage, anorexia, and diarrhea. On necropsy, septicemic lesions were found, general congestion of the internal organs, punctiform hemorrhages of the serous membranes, hypertrophy of the liver, and swelling of the spleen with subcapsular hemorrhages. The spiral organism was found in heart blood, spleen, liver, kidney, lung, bone marrow, brain, and intestinal content. Also found was a species of Leucocytozoon. Carpano's description of Microspirillum sounds more like that of a spirochete since he said that the organism moves like a trypanosome, using its extremities like flagella and making revolving and twisting movements. He described the organism as being at most 6 μ in length and having up to four nuclear granules. He stated that it differed from *S. minus* in not having flagella.

Santos Dias [8] reported the finding of an organism, *Microspirillum fragae*, in smears from lungs of a plover-like bird (*Burhinus capensis capensis*, Lichtenstein, 1823) from Mozambique. The drawing illustrating his article shows organisms somewhat resembling the organism of the present report as seen in stained smears, except that his are smaller and have only up to four dark-staining bodies. He did not mention the dark field appearance of the organism.

Yakimoff [9] told of finding spirochetes in the buccal cavity of chickens, resembling *Spirochaeta dentium*. He mentioned, without citing the place of publication, a finding by Wolf of a Treponema-like organism in the mouth of chickens.

Reis and Nobrega [10] also told of the finding by Wolf [11] (probably the same as quoted by Yakimoff) of spirochetes in the mouth cavity of geese, ducks, hens, and pigeons.

Various spiral organisms have been found causing disease in poultry in the United States, such as Borrelia in turkeys [12] and in pheasants,[13] and Spironema in chickens, turkeys, and pheasants.[14] None of these has been described as causing a stomatitis.

With the probable exception of the organisms found by Krage and Weisgerber [4] and the possible exception of those found by Jasmin,[1-3] the spiral organisms found causing disease in poultry have been spirochetes as distinguished from spirilla. Bergey [15] makes the distinction, the chief differences being the rigid nonflexible cell structure with flagella as locomotor organs in the spirilla, as compared to the flexible cell structure with no apparent flagella in the spirochetes.

Conclusion

The spiral organism of the present report is evidently to be classed as a Spirillum, since there is a rigid inflexible body, not like the flexuous body of the spirochetes. This Spirillum has a flagellum at either end, rather than the tufts of flagella described for most species of Spirillum. In this respect, the organism resembles *Spirillum minus*, although it is of larger size.

Although the Spirillum could not be obtained in pure culture by the methods used, it seems likely that it was the causative agent of the lesions described. This opinion is based on: (1) the finding of the organism in birds having the lesions and not in other birds; (2) the finding of the organism in great numbers in material taken directly from lesions, whereas fewer spirilla could be seen in smears made from other parts of the mouth; and (3) the finding of the Spirillum in lesions produced in experimentally inoculated birds.

Apparently, this Spirillum has not been observed previously. The name, *Spirillum pulli*, species *nova*, is hereby proposed.

Summary

A disease of chickens, evidenced by diphtheritic lesions of the buccal mucous membrane is reported. The apparent causative agent is a spiral organism, *Spirillum pulli*, species *nova*. This Spirillum, found in lesions and salivary glands, has not been cultured but can be transmitted from bird to bird by contact and by experimental inoculation.

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Studies of Infectious Synovitis in Chickens

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In 1954, Olson *et al.*[1,2] and Wills[3,4] reported an unidentified agent which produced a synovitis in chickens affecting primarily the synovial or bursal membranes. Cover *et al.*[5,6] and Leece and Sperling[7] reported on the same or a similar disease. Snoeyenbos and Olesiuk[8] observed a natural outbreak of the same or a similar disease in turkeys. Among the several terms applied to the disease, the following have been used most frequently: enlarged joint condition,[1,2] arthritis,[3-5,10] infectious anemia-synovitis,[11] and tendovaginitis or arthritis.[7] The term infectious synovitis suggested by Wills[4] is used in this paper.

Wills and Delaplane[9] reviewed the literature on the etiology of arthritis. Hofstad[14] mentioned the following bacteria as a cause of an arthritic condition in chickens: *Streptococcus gallinarum, Streptococcus zooepidemicus, Staphylococcus pyogenes, Bacterium arthropyogenes, Escherichia venezuelensis, Escherichia coli,* and *Salmonella pullorum.* Arthritis has been experimentally produced by pleuropneumonia-like organisms (PPLO).[10,15,16] Olson[17] isolated PPLO from swollen hocks and breast blisters of chickens presented to him for diagnosis. The etiology of infectious synovitis described in this paper is not related to previously described arthritic conditions caused by a PPLO or bacteria.

History

The disease has appeared in both broiler and replacement flocks. In West Virginia, during 1954, it was recognized in 55 flocks. The flocks varied in size from 1,000 to 20,000 birds. In 21 flocks, containing 154,000 birds, the approximate mortality from this disease was 7,700. The morbidity ranged from 1 to 75 per cent. The highest morbidity occurred in flocks in which a respiratory infection, or the hemorrhagic syndrome, or both were complicating factors.

The first observable signs in affected flocks were: pale comb, lameness, and retarded growth. Many flock owners have mistaken these signs for those of coccidiosis. As the disease progressed, the feathers became ruffled and the comb shrunk. In some cases, the comb was bluish red. Swellings usually occurred around the joints and breast blisters were common. The hock joints and foot pads were principally involved but, in some birds, all joints became affected. However, it was not uncommon to find birds with generalized infection but without apparent swelling of the joints. The birds became listless, dehydrated, and emaciated (fig. 1). Although birds were severely affected, many continued to eat and drink if placed near feed and water. A greenish discoloration of the droppings, which contained large amounts of uric acid or urates, was frequently observed.

Necropsy of birds in the early stages of the disease revealed a viscous, creamy exudate in the synovial membranes of affected joints, tendon sheaths, and bursal membranes of the keel. As the disease progressed, this yellow exudate became caseous. Caseous exudate was also occasionally found over the skull and along the neck. Where the birds were severely emaciated and dehydrated, there was occasionally no fluid about the joints. In chronic cases, the joint surface was frequently yellow to orange.

In the early stages of the disease, the spleen was frequently enlarged two to three times; however, in advanced stages, the spleen was usually not enlarged. The liver frequently appeared normal. In acute cases, the liver was generally enlarged and mottled, contained necrotic foci, and was greenish. The kidneys were usually swollen, mottled, or pale.

Giemsa stains of smears made from the creamy synovial exudate revealed many large mononuclear cells, lymphocytes, plasma cells, and heterophils. In some cases, there were more heterophils than mononuclear cells. No bacteria were found.

A decrease in the number of erythrocytes and an increase in leukocytes was commonly found in affected birds. The hemoglobin values were usually below normal, falling to a low of 3.5 Gm. per 100 ml. of blood. Blood smears made from field and experimentally infected birds revealed a slight to marked anemia. The erythrocytes appeared achromic in nearly all cases with some cells showing polychromatophilia. Slight to marked anisocytosis and poikilocytosis were also seen. Immature erythrocytes were frequently ob-

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TABLE 1—The Effect of Infectious Synovitis on Hemoglobin Content and Red and White Cell Counts* of Blood from Infected Chickens

Degree of infection	Source of chickens	No. of chickens	No. of counts	r.b.c. (millions/mm.³)	w.b.c. (thousands/mm.³)	Hemoglobin (Gm./100 ml. blood)
None[a]	Experimental	65	65	2.22	40.56	8.57
Third[b]	Experimental	12	12	1.67	78.83	7.28
Fourth[c]	Experimental	31	31	1.68	80.81	6.5
Fourth	Field	24	78	2.01	66.93	6.84
Unknown	Field	16	16	1.9	43.3	8.37

* The method of Natt and Herrick[13] was used for making counts.
[a] Contact controls. [b] Localized infection in the foot pad. [c] Generalized infection.

served. Usually, there was an increase, sometimes quite marked, in the percentage of monocytes and heterophils with a corresponding decrease in the percentage of lymphocytes. In some smears, numerous immature leukocyte "preforms" were present, namely, promonocytes, prolymphocytes, and myelocytes. The blast forms of these cells occurred occasionally. The changes in the blood picture seemed to vary directly with the severity of the infection as shown (tables 1 and 2).

EXPERIMENTAL DATA

The synovitis agent was cultured in chicken embryos. This was accomplished by inoculating the yolk sac of 5-day-old embryonating eggs with a broth suspension of the joint exudate, liver, or spleen from an infected bird. Attempts to grow the agent in blood, nutrient, and Sabouraud's maltose agar medium, thioglycollate medium, or nutrient broth were unsuccessful. Only a faint zone of hemolysis occurred on the blood agar. When cultures of the synovitis agent in eggs were placed in PPLO broth (Difco) containing either 2 per cent serum fraction or 20 per cent horse serum, no cloudiness of the broth occurred through 16 passages. The sixth broth culture transfer of two isolates was inoculated into the yolk sac of embryonating eggs with negative results. Growth could not be demonstrated in Adler's RM medium[12] or on PPLO agar.

Embryo mortality, through 20 passages of one isolate, occurred according to the pattern shown (table 3). For the first five passages, the "median of mortality" was

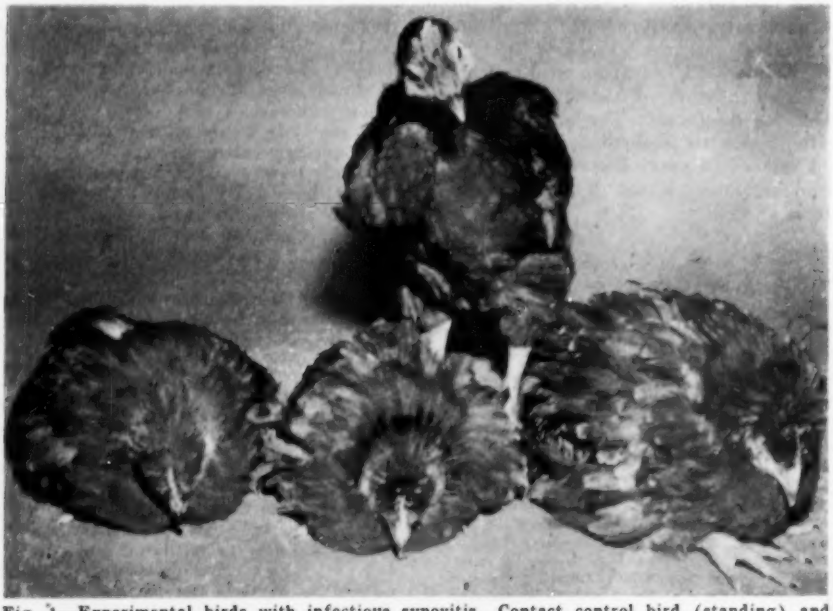

Fig. 1—Experimental birds with infectious synovitis. Contact control bird (standing) and 3 typically infected birds in the advanced stage of the disease.

TABLE 2—The Effect of Infectious Synovitis on Differential White Blood Cell Counts of Blood from Infected Chickens

Degree of infection	Source of birds	No. of birds	No. of counts	Lympho-cytes	Hetero-phils	Mono-cytes	Eosino-phils	Baso-phils	Pre-forms
None[a]	Experimental	65	65	66.09	22.46	8.92	0.89	1.12	0.79
Third[b]	Experimental	12	12	55.42	25.75	17.83	0.01	0.58	0.25
Fourth[c]	Experimental	31	31	41.87	31.94	19.29	0.23	1.06	6.07
Fourth[c]	Field	24	72	36.2	48.2	11.0	0.32	0.3	3.9
Unknown	Field	16	16	43.9	35.0	15.6	2.1	0.68	2.8

[a] Contact controls. [b] Localized infection in the foot pad. [c] Generalized infection.

ten days and, for the next 15 passages, it was eight days. Embryos which died during the first two days following inoculation were not considered in determining this median. Of 108 eggs inoculated during these 20 passages, the embryos of 97 died, 5 lived but revealed pathological changes, 3 were normal, and 3 were accidentally destroyed prior to observation. Ten isolates from different flocks have shown a similar embryo mortality pattern.

The agent has been propagated for 37 passages in the yolk sac of embryonating chicken eggs without a marked increase in its pathogenicity for embryos.

When 5-day-old embryonating chicken eggs were inoculated via the yolk sac, mortality occurred as early as the fourth day following inoculation. The embryos which died four to ten days following inoculation were edematous. There were numerous petechial hemorrhages of the skin which tended to coalesce (fig. 2). The liver was generally enlarged, frequently hemorrhagic, and mottled. The spleens and kidneys were usually enlarged and hemorrhagic. External hemorrhages were not commonly found in embryos dying after the tenth day following inoculation. In the older embryos, the spleen was frequently enlarged as much as six times. The allantoic fluid was frequently cloudy. Occasionally, cloudy fluid was present in the abdominal cavity and pericardial sac. Slight dwarfing of the embryos occurred. Caseous exudate in the air sacs was not observed.

The route of inoculation was important if the agent was to be propagated. When 9-day-old embryonating eggs were inoculated with joint exudate into the allantoic cavity, no embryo mortality occurred. Eighteen days later, material from the same source was inoculated into the yolk sac of 6-day-old embryonating eggs and a typical embryo mortality pattern resulted. Internal organs, yolk, and allantoic fluid were taken from dead infected embryos and inoculated into the yolk sac of 6-day-old embryonating eggs. A series of five passages was made. All inoculums were infective for embryos for the first three passages. The fourth and fifth embryo passage of allantoic fluid was not infective, whereas the yolk and ground internal organs produced infection of embryos. Inoculation of infective material by the yolk sac route was found to be the most desirable for culture of the agent in eggs.

The growth of the agent in embryonating eggs was inhibited by streptomycin but not by penicillin. A 1:10 dilution of infected yolk was treated with 10,000, 20,000, 30,000, and 40,000 units of penicillin per milliliter and 25, 50, and 75 mg. of streptomycin per milliliter. The treated yolk was allowed to stand at room temperature for two hours and inoculated, in 0.25-ml. quantities, into the yolk sac of four 6-day-old embryonating eggs. All

TABLE 3—Embryo Mortality Pattern Following Inoculation of Eggs with Agent 36[a]

Passage	No. eggs inoculated	Death following inoculation					Lived
		1 to 5 days	6 to 7 days	8 to 9 days	10 to 11 days	12 to 13 days	
1 to 5	25	0	5	4	9	4	3
6 to 10	24[†]	3	7	5	5	2	1
11 to 15	21[†]	3	6	3	2	3	3
16 to 20	38[†]	3	12	12	6	3	1
Total	108	9	30	24	22	12	8

[a] Yolk (0.25 ml.) taken from infected embryos was introduced into the yolk sac of 5-day-old embryonating chicken eggs.

[†] One embryo in the group was accidentally destroyed.

embryos which were inoculated with penicillin-treated material died, whereas all embryos inoculated with the streptomycin-treated material survived.

A yolk culture of the synovitis agent stored in a commercial-type deepfreeze at —20 C. was active up to 271 days. The pathogenicity for embryos was, however, reduced. Joint fluid from infected chickens diluted 1:5 with nutrient broth was not infective for chickens after 22 days' storage at —20 C. Similar fluid from another source was infective for chickens and embryos after 76 days' storage, although the pathogenicity of the agent was greatly reduced.

Inconsistent results were obtained in filtration trials. When infective yolk was diluted 1:10 with nutrient broth, the agent was recovered from the filtrate obtained with a Broener-type microfilter. The filtrate obtained from the same source with a Seitz EK filter was inactive. Leece and Sperling[7] reported that filtering the agent through a sintered glass filter markedly decreased the infectious titer. Electron micrographs made by Leese and Sperling[7] of allantoamniotic fluid revealed coccobacillary elements, 0.2 to 0.5 μ in diameter.

Transmission Trials.—The infectious synovitis agent has been isolated from 15 flocks but most experimental studies have been made on isolations from one flock of 11,000 7-week-old broilers. The disease in this flock was complicated with a respiratory infection and the hemorrhagic syndrome. The morbidity in this flock was approximately 75 per cent and mortality 10 per cent. The synovitis agent was isolated from hock exudate and tracheal mucus from 1 bird and these isolates will be referred to as 29H and 29M, respectively. In another bird from the same flock, the synovitis agent was isolated from the hock exudate and it will be referred to as agent 36.

Fig. 2—Two 15-day-old embryos. The infectious synovitis-inoculated embryo on the left is edematous and has numerous hemorrhages in the skin. The embryo on right is normal.

Transmission of infectious synovitis by the oral route was attempted. A suspension of ground livers and spleens from infected chickens in nutrient broth was fed to chicks daily for eight days and followed by another dose on the fourteenth day. The 13 4-week-old chicks remained normal during the period of treatment and for the subsequent 13 days. Six birds from the group were then inoculated with the first or second chicken passage of agent 36 via the foot pad and all developed signs of synovitis.

The inoculum used for the chicken transmission trials consisted of a broth suspension of joint exudate from bird 36 (agent 36) or from bird 29 (agent 29H). The suspension was inoculated into chickens by the various routes listed below. In the description which follows, the numerator represents the number of chickens which became infected and the denominator the number of chickens which were inoculated: nasal instillation, 0/3; conjunctival instillation, 1/4; tracheal instillation, 0/1; foot pad inoculation, 2/2; hock joint inoculation, 6/6; and conjunctival and nasal instillation, 1/1. The first and second chicken passages were inoculated in a similar manner with the following results: foot pad inoculation, 6/6; hock joint, 4/4; and air sac, 1/1. No infection occurred in 14 contact controls during a 25- to 44-day observation period.

Cultures of the synovitis agent (29M, 29H, and 36) in embryonating eggs were also pathogenic for chickens. The concentration of the agent in allantoic fluid was apparently low; however, yolk, ground internal organs, and ground embryo tissues were pathogenic for chickens. Since the yolk was easier to handle and appeared to maintain its infectivity longer in stor-

age, embryo yolk cultures were used for most of the transmission trials.

The following embryonating egg cultures of the synovitis agent have been used for chicken inoculation: first, second, third, fifth, tenth, eighteenth, twenty-first, and twenty-fifth passages. All were pathogenic for chickens. The third embryo yolk culture of the synovitis agent was inoculated into chickens in 0.25-ml. quantities with the following results: foot pad, 16/16; vein, 5/15; sinus, 5/15; conjunctival instillation, 0/4; and contact controls, 3/22. The results of the second and twenty-fifth embryo-passage inoculations are given (table 4). The other embryo yolk cultures were inoculated into the foot pads of chickens with the following results: first, 1/2; fifth, 3/8; tenth, 5/5; fourteenth, 2/5; eighteenth, 4/4; and twenty-first, 2/7. The infectivity for chickens of embryo cultures could be increased by passing the agent through chickens.

The incubation period in experimentally affected chicks was governed by the route of inoculation, amount of inoculum, and the virulence of the agent. Most inoculations were made in birds 3 to 6 weeks of age. After inoculation in the pad of the foot, swellings of the part usually occurred within two to ten days. Birds inoculated intravenously or intracranially became droopy in seven to ten days, with death occurring in ten to 14 days and often occurring before swellings appeared around the joints; however, a small amount of exudate was usually present about the hock or pad joints. The incubation periods, in days, after other routes of inoculation were as follows: sinus, 14 to 20; air sac, seven to 14; and conjunctival instillation, 20. In contact control chickens, the incubation period was 25 to 80 days.

Spontaneous recovery was rare in experimental birds. Localization of infection in the pads of some inoculated birds suggested that they might have recovered if they had not been killed.

Observations of infectious synovitis in the field suggested that transmission was possible while the chicks were in the hatchery. The infection occurred in a broiler flock in a newly constructed house on a farm where chickens had not been previously raised. Broiler producers, who obtained chicks from two hatcheries at the same time, had infections in chicks from one hatchery but not in those from the other. The first 8 cases of infectious synovitis brought to our attention were from the same hatchery. Later, birds from other hatcheries were found to be infected.

From the suspected hatchery, approximately 75 chicks from pipped eggs and 75 cull chicks were examined but no enlarged spleens and livers were found. Also, 52 dead embryos from 13 supply flocks were removed on the eighteenth day of incubation. Of the 52 embryos, 30 were decomposed and were discarded. The allantoic fluid, yolk, or liver and spleen of the other embryos was harvested and inoculated into the yolk sac of 6-day-old embryonating eggs. Seven of these eggs became contaminated and were discarded. An agent was recovered from one of the remaining eggs and it was carried through 15 egg passages. Inoculation of embryonating eggs with this agent resulted in erratic mortality. The embryos died during the fourth to thirteenth days following inoculation. Those dying later were dwarfed. The agent involved has not been identified. It is unlikely that it is related to the infectious synovitis agent, since none of the embryos which died revealed typical pathological changes.

Ten dozen eggs were obtained from a flock of approximately 4,000 birds which had a previous history of infectious synovitis. At the time the eggs were obtained, the birds had been housed for one month

TABLE 4—The Susceptibility of 3-Week-Old Chicks to Embryo Cultures of Three Isolates of the Infectious Synovitis Agent*

Route of inoculation	Isolate	Infected	Percentage infected	Deaths
Foot pad[1]	36	4	100	0
	Roy	4	100	3†
	29M	4	100	4†
Sinus[1]	36	1	25	1†
	Roy	1	25	1†
	29M	2	50	2†
Brain[2]	36	0	0	0
	Roy	1	25	1
	29M	4	100	4†
Muscle[1]	36	0	0	0
	Roy	1	25	1
	29M	4	100	3†
Contact control	36	0	0	0
	Roy	0	0	0
	29M	1	25	1†

* Embryo passage used: 36, twenty-fifth; Roy, second; 29M, second.
† One bird killed.
[1] Inoculum consisted of 0.25 ml. of yolk embryo culture. [2] Inoculum consisted of 0.10 ml. of yolk embryo culture.

and those showing enlarged joints had been culled. The eggs were incubated and candled daily. There were 28 infertile eggs and 16 fertile eggs, the embryos of which died at an early age. Yolk was taken from these eggs and inoculated into the yolk sac of 6-day-old embryonating chicken eggs. No agent was recovered from these 44 eggs. Embryos of six eggs were dead at 21 days and seven of the eggs had been pipped. None was abnormal nor did the yolks yield an infectious agent. The remaining eggs were allowed to hatch; the chicks remained normal during a four-week observation period.

TABLE 5—Susceptibility of Turkeys to Infectious Synovitis Agent 36*

Inoculum	Intravenous inoculation†	Foot pad inoculation†
18th embryo passage		2/2ª/3
1st chicken passage	3/3/3	3/3ª/3
2nd chicken passage		3/2/3
3rd chicken passage	3/0/3	1/0/1
Nutrient broth	0/0/1	
Normal yolk		0/0/2
Contact controls	0/0/2	0/0/2
Isolated controls	0/0/4	

* Amount of inoculum used was 0.25 ml.
† Number showing symptoms or lesions/number died/number inoculated. a Killed.

Yolk cultures of agent 36 were diluted from 10^1 to 10^5 with physiological saline solution and inoculated into 5-day-old embryonating chicken eggs. Three eggs were used for each dilution. The embryos which survived were allowed to hatch. The chicks were killed and examined at seven days. The spleens of 2 were enlarged, one from the 10^2 the other from 10^4 dilution. The agent was recovered from the spleen of a chick receiving the 10^2 dilution. Wills [4] inoculated a hock exudate suspension into the yolk sac of 11 7-day-old embryos. Of 7 normal chicks obtained from the 11 eggs, 1 developed arthritis on the nineteenth day. No lesions were found in the remaining chicks when 4 weeks old.

Susceptibility of Other Animals.—Attempts were made to transmit agent 36 by intramuscular inoculation of 12 young Swiss mice; by intraperitoneal inoculation of a 5-day-old lamb, 2 5-day-old pigs, and 5 3-day-old rats; by intracranial inoculation of 5 3-day-old rats; and by intramuscular and subcutaneous inoculation of 1 rabbit. In all cases, the inoculum had been found to be pathogenic for chickens. Neither the lamb, rabbit, mice, rats, or pigs developed signs of infectious synovitis nor were gross lesions of infectious synovitis observed at necropsy.

The susceptibility of turkeys to infectious synovitis was ascertained by exposing 4-week-old poults to the eighteenth yolk embryo passage and first, second, and third chicken passages of a joint fluid suspension of agent 36. The results are summarized (table 5).

The turkeys were kept in isolation cages

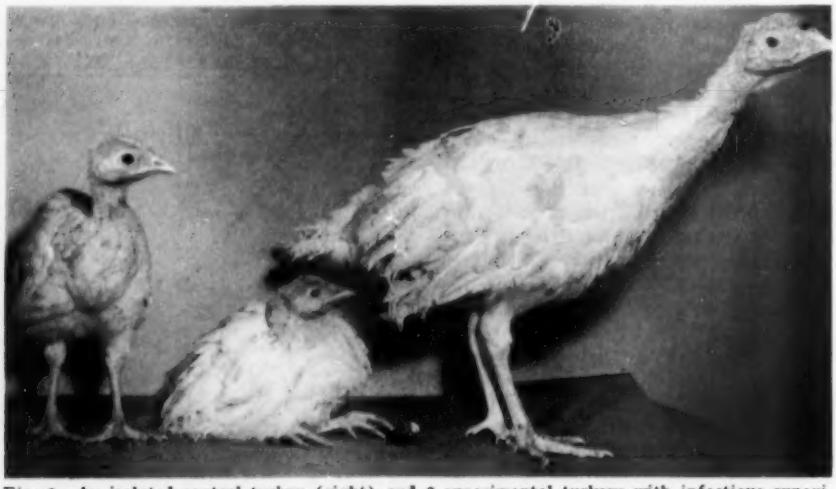

Fig. 3—An isolated control turkey (right) and 2 experimental turkeys with infectious synovitis (left).

for 30 to 35 days following inoculation and necropsies were done on those with signs of infectious synovitis. The remaining turkeys were moved to an outside house and observed for an additional 60 days. All turkeys placed outside remained normal.

Turkeys experimentally infected with infectious synovitis had essentially the same signs, lesions, and incubation period as those described for chickens (fig. 3).

The synovitis agent was recovered in embryonating chicken eggs from experimentally inoculated turkeys by the methods previously described in this paper.

Snoeyenbos and Olesiuk [8] reported on an agent isolated from naturally occurring arthritis in turkeys. The agent was more pathogenic for chickens than for turkeys. These workers believed the agent to be similar to the one reported by Wills [3, 4, 10] and to the synovitis agent described in this report.

Discussion

A diagnosis of infectious synovitis can usually be made on the basis of pale combs, droopiness, emaciation, leg weakness along with breast blisters and enlarged foot pads, or hock joints which contain a viscous creamy or caseous exudate. Bacteria and PPLO as a cause of synovitis or arthritis must be eliminated by bacteriological procedures. Where possible, yolk sac inoculation of 5- or 6-day-old embryonating eggs should be made.

The reports of Wills,[4, 9] Snoeyenbos and Olesiuk,[8] Cover and Galeta,[5] Cover *et al.*,[6] and personal communications from various diagnostic laboratories and poultry processors indicate that the disease is widespread in the broiler-producing areas of the United States.

Infectious synovitis described in this report is a separate disease entity caused by a large-particle virus or viruses. This conclusion is based on the lack of growth in cell-free mediums, the ability of the agent to pass a Sietz filter,[6] susceptibility to antibiotics,[18–20] and particle size as determined by electron photomicrographs (Leece and Sperling [7]). Whether the disease is caused by a single virus or several viruses must be determined by further research on infectious synovitis. The importance of a PPLO synovitis [17] must be established.

Infectious synovitis of chickens is characterized by anemia, pale comb, leg weakness, droopiness, emaciation, enlarged hocks and foot pads, breast blisters, and dehydration; at necropsy, by creamy or caseous exudates involving the synovial or bursal membranes, swollen kidneys, occasionally enlarged and discolored livers, and occasional splenomegaly; and hematologically, by reduced hemoglobin, lower erythrocyte and higher leukocyte counts, by decrease in the percentage of lymphocytes, and by an increase in the percentage of monocytes, heterophils, and "preforms" of white blood cells.

Summary

The signs, lesions, transmission, cultivation, and susceptibility of other animals to infectious synovitis of chickens are described.

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Electrophoretic and Serum Neutralization Studies of Infectious Bronchitis of Chickens

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ELECTROPHORETIC and chemical analyses of antibacterial and antiprotein serums show, in general, a decrease of albumin and an increase of globulins, particularly *gamma* globulin.[1-3] There have also been reports to the contrary.[4,5] Some antiviral serums of high antibody content, such as those of western and Venezuelan equine encephalomyelitis, Japanese B encephalitis, influenza, foot-and-mouth disease, and vesicular stomatitis, exhibit little or no change from the normal with respect to albumin and globulin.[2,6-13] Other antiviral serums, particularly those of infectious hepatitis, homologous serum jaundice, infectious mononucleosis, lymphogranuloma, and equine infectious anemia, in which antibody studies have been conducted, show low albumin and high globulin values.[2,14-21] The present study was undertaken to determine any quantitative relationship between total globulin, *gamma* globulin, and neutralizing antibodies as shown by electrophoretic and serum-neutralization studies of serums from chickens exposed to infectious bronchitis virus (IBV).

MATERIALS AND METHODS

TWO different strains of IBV were employed. Strain VR (lot 285),* a chicken-propagated strain, was used in the form of an infected lung and tracheal suspension to inoculate the chickens. Signs of the disease usually appeared about 24 hours after intratracheal instillation of 0.2 ml. of the suspension followed by swabbing of the trachea.

Strain V114D, a chicken embryo-adapted strain capable of killing all embryos within 36 hours after inoculation via the allantoic cavity, was used as the antigen in the serum-neutralization tests. Infectious bronchitis virus antibodies specifically neutralized this strain.

Single comb White Leghorn cockerels,† ranging in age from 7 to 9 months at the beginning of the experiment, were used. The chickens were divided into three groups as follows: group 1, controls; group 2, chickens receiving one inoculation with IBV; and group 3, chickens inoculated with IBV and reinoculated 12 weeks later.

A commercial, growing mash containing at least 20 per cent protein was fed *ad libitum*. The chickens were fasted for 18 to 24 hours prior to the collection of 20 ml. of blood by cardiac puncture at each bleeding period. All serums were negative for Newcastle disease antibodies as determined by the hemagglutination-inhibition test. All chickens which died during the course of the experiment and those living at the terminal period were subjected to necropsy.

For the serum-neutralization test,[22,23] serial tenfold dilutions of IBV, V114D-infected allantoic fluid were prepared in Difco nutrient broth and mixed with equal portions of undiluted serum. Quantitative virus controls were prepared by mixing equal portions of each virus dilution with Difco nutrient broth. All mixtures were incubated at 4.0 C. for 30 minutes. Five 10-day-old embryonating chicken eggs were used per dilution and 0.1 ml. of inoculum was injected via the allantoic cavity. All incubation was at 99.0 to 99.5 F., wet bulb 86.0 to 88.0 F., in an electric, forced-draft incubator. Embryo mortality within the first 18 hours postinoculation was considered to be due to nonspecific causes and was not included in the final computations. The postinoculation observation period was five days. The lethal dose$_{50}$ neutralization index ($I.d._{50}$ NI) of each serum was calculated according to the method of Reed and Muench.[24]

A standard veronal buffer[25] of 0.1 ionic strength, pH 8.6, as measured with the glass electrode at 25.0 C., was used for dilution and dialysis of the serums prior to electrophoretic analyses. Each serum was analyzed for protein by the Kjeldahl method, diluted to a 2 per cent concentration, and then dialyzed according to the method of Reiner and Fenichel.[26] Electrolytic conductance equilibrium, as measured at 0.5 C., was attained after dialysis for 90 minutes at 25.0 C.

The Perkin-Elmer-Tiselius electrophoresis apparatus, model 38, was used. The current was adjusted to 6 ma. and the voltage was maintained

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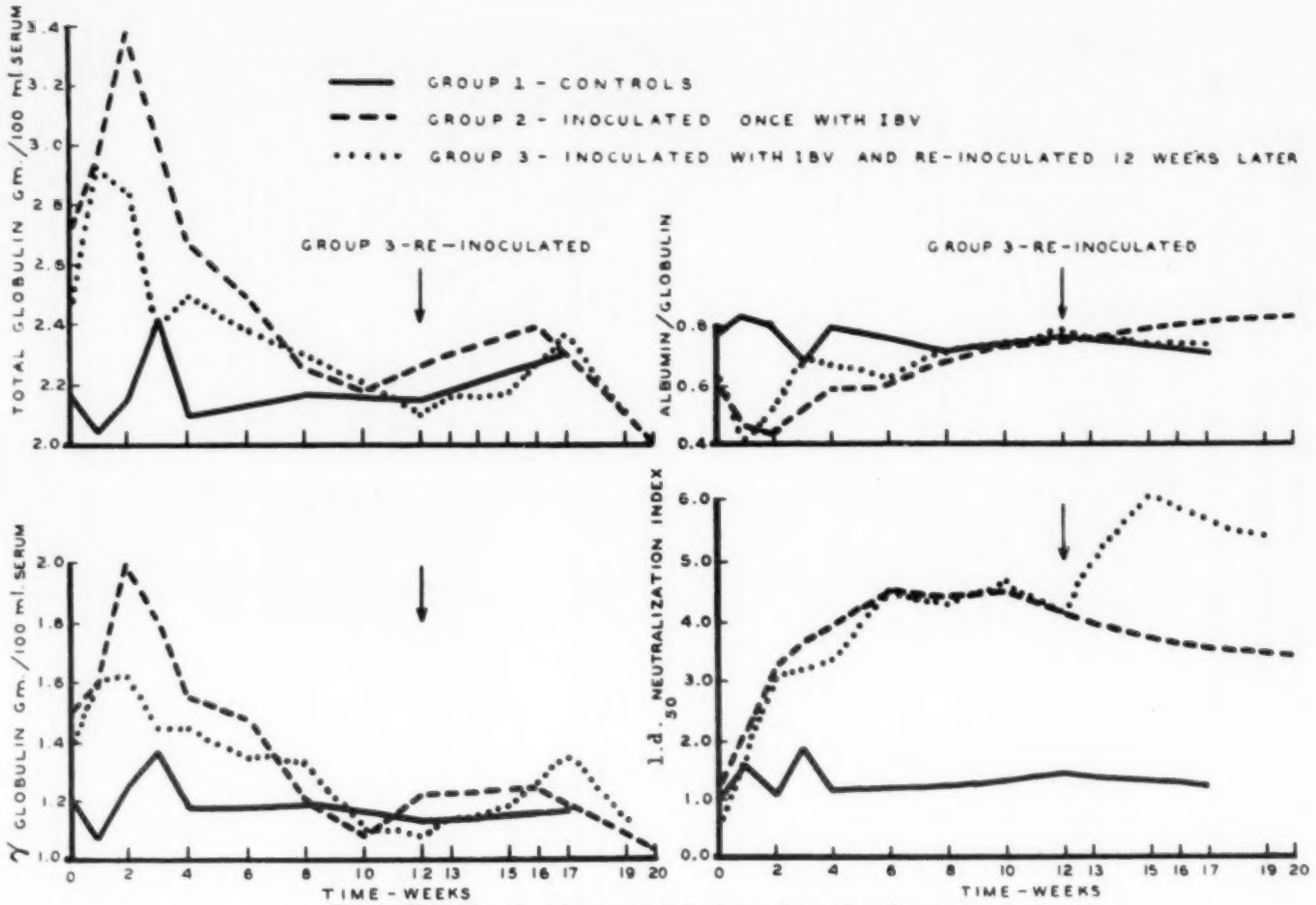
GROUP 1 - CONTROLS
GROUP 2 - INOCULATED ONCE WITH IBV
GROUP 3 - INOCULATED WITH IBV AND RE-INOCULATED 12 WEEKS LATER
TOTAL GLOBULIN GM./100 ml SERUM
GROUP 3-RE-INOCULATED
ALBUMIN/GLOBULIN
γ GLOBULIN GM./100 ml SERUM
l.d.50 NEUTRALIZATION INDEX
TIME - WEEKS
TIME - WEEKS

Fig. 1—Electrophoretic and serum-neutralization analyses of chicken serum.

at approximately 107.0 v., giving a potential gradient of approximately 6.5 v. per centimeter[2] at 0.5 C. in a 2-ml. capacity electrophoresis cell having a cross sectional area of 0.3 cm.[2] When the fastest moving component had migrated about two thirds the distance through the cell (7,600 seconds), the separated serum components were photographed by the Longsworth scanning modification of the *schlieren* system.[27]

The pattern from the descending limb was enlarged twofold and traced on paper. The areas that were attributable to the various serum components, as defined by Tiselius and Kabat,[28] were measured with a planimeter and the concentrations computed. Mobilities were calculated according to the method of Longsworth and MacInnes.[29]

Results

The pertinent information to be derived from the electrophoretic analyses is best presented in summation as the albumin-globulin ratio (A/G), total globulin and *gamma* globulin as grams per 100 ml. of serum (G Gm./100 ml. and γG Gm./100 ml., respectively), and $l.d._{50}$ NI for the individual chickens, and the average for each group as shown (tables 1, 2, and 3). Since not all chickens participated at each sampling period of the experiment and marked individual variations were found, assessment of the results is best approached through consideration of the trends as shown by the average data for each group. These average data are presented graphically (fig. 1).

Group 1, Controls.—Variations encountered in the A/G for the group were most prominent during the first four weeks of the experiment and reflected the fluctuations of G and γG when samples were collected weekly. During the remaining portion of the experiment when samples were collected at intervals of four weeks or more, the A/G was relatively stabile and of the same general magnitude as the initial value (table 1, fig. 1). With the exception of chicken 4 at three periods, all A/G values showed a greater concentration of globulin than albumin.

Total globulin and γG values varied at the several sampling periods but a general trend was evident. The initial averages of G 2.17 Gm./100 ml. and γG 1.22 Gm./100 ml. decreased in one week to 2.04 and 1.07, respectively. These were followed by progressive increases to maximums of 2.42 and 1.37 for the third week. At the fourth week, total globulin was 2.09 Gm./100 ml. but, for the eighth and twelfth weeks, it

TABLE 1—Electrophoretic and Serum-Neutralization Analyses of Chicken Serum of Group 1—Controls

No.		Time (weeks) 0	1	2	3	4	8	12	17
1*	A/G†	0.69	0.77	0.65	0.64	0.7	.....	0.62	.....
	G	2.24	2.21	2.5	2.57	2.27	.....	2.56	.....
	γG	1.28	1.23	1.47	1.5	1.27	.....	1.44	.....
	NI	0.9	1.9	1.1	1.8	1.7	.....	1.3	.....
2	A/G	0.84	0.85	0.91	0.68	0.66‡	.....	.....	.....
	G	2.13	1.95	1.84	2.4	2.32	.....	.....	.....
	γG	1.27	1.02	1.11	1.39	1.36	.....	.....	.....
	NI	0.9	1.1	1.6	1.9	0.5	.....	.....	.....
3	A/G	0.73	0.89	0.88	0.64‡	.....	.....	.....	.....
	G	2.15	1.97	2.15	2.3	.....	.....	.....	.....
	γG	1.1	0.95	1.16	1.22	.....	.....	.....	.....
	NI	1.0	1.5	0.3	1.7	.....	.....	.....	.....
4	A/G	.....	.....	.....	.....	1.03	1.07	0.96	1.03
	G	.....	.....	.....	.....	1.69	1.66	1.75	1.77
	γG	.....	.....	.....	.....	0.92	0.87	0.82	0.84
	NI	.....	.....	.....	.....	1.1	1.8	1.5	1.0
5	A/G	.....	.....	.....	.....	.....	0.56	.....	0.47
	G	.....	.....	.....	.....	.....	2.7	.....	3.17
	γG	.....	.....	.....	.....	.....	1.45	.....	1.62
	NI	.....	.....	.....	.....	.....	1.3	.....	0.7
6	A/G	.....	.....	.....	.....	.....	0.64	.....	0.76
	G	.....	.....	.....	.....	.....	2.14	.....	2.0
	γG	.....	.....	.....	.....	.....	1.24	.....	1.06
	NI	.....	.....	.....	.....	.....	0.6	.....	1.8
Av.	A/G	0.75	0.83	0.8	0.66	0.78	0.71	0.76	0.7
	G	2.17	2.04	2.16	2.42	2.09	2.17	2.15	2.31
	γG	1.22	1.07	1.25	1.37	1.18	1.19	1.13	1.17
	NI	0.9	1.5	1.0	1.8	1.1	1.2	1.4	1.2

* This sample was a pool of equal portions of serums from chickens 5 and 6.

† A/G = albumin-globulin ratio; G = total globulin as grams per 100 ml. of serum; γG = *gamma* globulin as grams per 100 ml. of serum; NI = $l.d._{50}$ neutralization index.

‡ Died after collection of blood.

was similar to the initial level with an increase to a terminal value of 2.31 Gm./100 ml. The level of *gamma* globulin from the fourth through the seventeenth weeks was lower than the initial level but remained relatively stabile (table 1, fig. 1).

The average of G and γG for chickens 5 and 6 at the eighth and seventeenth weeks, periods for which analyses had not been made on the pooled sample from these chickens, were in agreement with the value for the fourth and twelfth weeks and fit the general trend.

The average $l.d._{50}$ NI showed variation at the sampling periods but were in fairly close agreement with the normal acceptable level[23] of $10^{1.517} \pm 10^{0.0376}$. The most marked variations occurred during the first four weeks. During the remaining 13 weeks, the values were relatively stabile (table 1, fig. 1).

Chickens 2 and 3 died during the fourth and third weeks, respectively, from internal hemorrhage following collection of blood. At necropsy, they were normal and bacteriological and virological isolation attempts were negative.

Group 2, Chickens Inoculated Once with Infectious Bronchitis Virus.—The A/G decreased abruptly from 0.61 at the first week to 0.42 for the second week. From this time to the terminal period of 20 weeks, there was a constant progression to 0.82.

Total globulin and *gamma* globulin increased abruptly from 2.68 Gm./100 ml. and 1.49 Gm./100 ml., respectively, to maximums of G 3.38 Gm./100 ml. and γG 1.99 Gm./100 ml. in two weeks. These were followed by progressive decreases to G 2.18 Gm./100 ml. and γG 1.08 Gm./100 ml. for the tenth week. Both showed minor increases during the twelfth and sixteenth weeks followed by decreases to minimums of G 2.01 Gm./100 ml. and γG 1.03 Gm./100 ml. for the terminal period of 20 weeks. Subsequent to the fourth and sixth weeks, respectively, the levels of G and γG were below the initial level (table 2, fig. 1).

All $l.d._{50}$ NI at the beginning of the experiment were within the normal acceptable level.[23] The neutralizing antibody response was typical of IBV infection.[30] There was an ascending progression of the average $l.d._{50}$ NI from 1.1 to a maximum of 4.5 which remained essentially the same from the sixth through the tenth weeks. After this, there was a progressive

TABLE 2—Electrophoretic and Serum-Neutralization Analyses of Chicken Serum of Group 2—Chickens Inoculated Once with Infectious Bronchitis Virus

No.		Time (weeks)										
		0	1	2	3	4	6	8	10	12	16	20
1	A/G*	0.77	0.48	0.58	0.58	0.67	0.58	0.66	0.68	0.73	0.74	0.73
	G	2.36	2.61	3.06	2.94	2.52	2.83	2.53	2.62	2.44	2.88	2.44
	γG	1.32	1.41	1.87	1.73	1.49	1.81	1.46	1.38	1.33	1.51	1.33
	NI	0.9	1.9	3.0	3.6	4.1	4.9	5.0	5.7	4.3	4.1	4.1
2	A/G	0.62	0.36	0.48	0.52	0.65	0.59	0.65	0.69	0.7	0.76	0.73
	G	2.66	3.54	3.37	3.06	2.66	2.43	2.45	2.45	2.27	2.34	1.83
	γG	1.5	2.0	2.0	1.86	1.64	1.43	1.28	1.32	1.22	1.24	0.98
	NI	1.0	1.9	2.7	3.0	3.3	4.6	4.6	5.2	4.2	3.9	3.4
3	A/G	0.69	0.53	0.5	0.64	0.63	0.64	0.58	0.7	.....	.....	.....
	G	2.21	2.43	2.7	2.68	2.32	2.52	2.45	2.37	.....	.....	.....
	γG	1.23	1.3	1.56	1.64	1.37	1.49	1.37	1.18	.....	.....	.....
	NI	1.2	3.3	3.6	3.6	4.8	5.1	4.7	4.5	.....	.....	.....
4	A/G	0.46	0.45	0.22	0.31	0.35	0.34†	.....	.....	.....	.....	.....
	G	3.48	3.32	4.38	3.47	3.71	3.46	.....	.....	.....	.....	.....
	γG	1.92	1.73	2.53	2.0	2.09	2.11	.....	.....	.....	.....	.....
	NI	1.4	1.8	3.3	4.0	4.5	5.7	.....	.....	.....	.....	.....
5	A/G	.....	.....	.....	.....	0.67	0.77	0.7	0.72	0.8	0.74	0.8
	G	.....	.....	.....	.....	2.21	1.98	2.17	1.99	2.1	2.53	2.2
	γG	.....	.....	.....	.....	1.17	1.09	1.03	0.89	1.12	1.38	1.09
	NI	.....	.....	.....	.....	2.8	3.6	5.1	4.2	3.9	3.8	3.7
6	A/G	.....	.....	.....	.....	.....	0.76	0.81	0.98	.....	1.02	1.11
	G	.....	.....	.....	.....	.....	1.74	1.7	1.46	.....	1.87	1.58
	γG	.....	.....	.....	.....	.....	0.94	0.86	0.62	.....	0.83	0.73
	NI	.....	.....	.....	.....	.....	3.2	2.8	2.7	.....	2.7	2.5
Av.	A/G	0.61	0.45	0.42	0.5	0.57	0.59	0.67	0.73	0.74	0.8	0.82
	G	2.68	2.97	3.38	3.04	2.68	2.49	2.26	2.18	2.27	2.4	2.01
	γG	1.49	1.61	1.99	1.81	1.55	1.48	1.2	1.08	1.22	1.24	1.03
	NI	1.1	2.0	3.2	3.6	3.9	4.5	4.4	4.5	4.1	3.6	3.4

* A/G = albumin-globulin ratio; G = total globulin as grams per 100 ml. of serum; γG = *gamma* globulin as grams per 100 ml. of serum; NI = $l.d._{50}$ neutralization index.

† Died after collection of blood.

decrease to the terminal value of 3.4. Maximum individual l.d.$_{50}$ NI occurred during the sixth to tenth weeks and were reflected in the plateau of the averages at these periods (table 2, fig. 1).

At the termination of the experiment, chicken 2 showed visceral lymphomatosis at necropsy. Chicken 4 died during the sixth week from internal hemorrhage following collection of blood and was found to have neural lymphomatosis. Bacteriological and virological isolation attempts on these chickens were negative. The L component, as described by Sanders *et al.*,[31] in serums examined electrophoretically from chickens affected with various forms of lymphomatosis was not present in the serums from these chickens.

Group 3, Chickens Inoculated with Infectious Bronchitis Virus and Reinoculated Twelve Weeks Later.—With this group, major emphasis was placed on the antibody response following reinoculation at the twelfth week. Chicken 1 participated in the entire experiment to serve for comparison with group 2 during the period prior to reinoculation. Chicken 2 was employed during the sixth and tenth weeks to further establish the period of maximum l.d.$_{50}$ NI after initial inoculation. Chickens 3 and 4 were used solely for the antibody response following reinoculation.

The A/G followed a pattern similar to that of group 2, with certain minor differences. The initial ratio of 0.66 decreased to a minimum of 0.38 in one week. From this time to the terminal period of 19 weeks, the ratio increased with some fluctuation to 0.79.

Total globulin and *gamma* globulin likewise exhibited a trend similar to that of group 2. Total globulin increased from 2.41 Gm./100 ml. to a maximum of 2.92 in one week. This was followed by a decrease to 2.11 Gm./100 ml. for the twelfth week, an increase to 2.38 Gm./100 ml. for the seventeenth week, and an abrupt decline to the terminal value and minimum of 2.08 Gm./100 ml.

Gamma globulin increased from 1.38 Gm./100 ml. to the maximum of 1.62 Gm./100 ml. in two weeks followed by a general decline to the minimum of 1.08 Gm./100 ml. at the twelfth week. This increased to 1.25 Gm./100 ml. during the seventeenth week and declined to 1.13 Gm./100 ml. for the nineteenth week.

Neutralizing antibody response of chickens 1 and 2 following initial inoculation was typical for IBV infection and closely paralleled the trend of group 2. The l.d.$_{50}$ NI increased from 0.4 to a maximum plateau of approximately 4.6 from the sixth to tenth weeks followed by a decline to 4.1 for the twelfth week. The effect of reinoculation at this period was quite marked. The l.d.$_{50}$ NI increased from 4.1 to a maximum for the experiment of 6.1 in three

TABLE 3—Electrophoretic and Serum-Neutralization Analyses of Chicken Serum of Group 3—Chickens Inoculated with Infectious Bronchitis Virus and Reinoculated Twelve Weeks Later

No.		Time (weeks)												
		0	1	2	3	4	6	8	10	12*	13	15	17	19
1	A/G†	0.66	0.38	0.51	0.69	0.66	0.61	0.72	0.76	0.78	0.76	0.87	0.79	0.76
	G	2.41	2.92	2.84	2.39	2.49	2.29	2.3	2.1	2.07	2.19	1.79	2.21	2.0
	γG	1.38	1.61	1.62	1.44	1.44	1.3	1.33	1.05	1.09	1.18	0.97	1.16	1.02
	NI	0.4	1.5	3.0	3.2	3.3	3.7	4.3	4.4	4.1	5.1	5.6	5.8	5.0
2	A/G	.....	.....	.....	.....	.....	0.63	.....	0.71	0.64	0.69	0.55	0.62	0.62
	G	.....	.....	.....	.....	.....	2.48	.....	2.31	2.31	2.44	2.71	2.76	2.34
	γG	.....	.....	.....	.....	.....	1.4	.....	1.17	1.24	1.38	1.6	1.57	1.31
	NI	.....	.....	.....	.....	.....	5.2	.....	4.8	4.3	5.8	7.0	5.8	5.2
3	A/G	.....	.....	.....	.....	.....	.....	.....	.....	0.83	0.75	0.84	0.77	0.89
	G	.....	.....	.....	.....	.....	.....	.....	.....	2.11	1.96	2.01	2.18	2.0
	γG	.....	.....	.....	.....	.....	.....	.....	.....	1.07	0.96	1.03	1.14	1.15
	NI	.....	.....	.....	.....	.....	.....	.....	.....	4.3	5.1	6.3	6.2	6.0
4	A/G	.....	.....	.....	.....	.....	.....	.....	.....	0.92	0.8	0.77	0.75	0.93
	G	.....	.....	.....	.....	.....	.....	.....	.....	1.97	2.05	2.18	2.36	2.0
	γG	.....	.....	.....	.....	.....	.....	.....	.....	0.93	0.99	1.12	1.12	1.05
	NI	.....	.....	.....	.....	.....	.....	.....	.....	3.8	4.0	5.5	5.2	5.2
Av.	A/G	0.66	0.38	0.51	0.69	0.66	0.62	0.72	0.73	0.79	0.75	0.74	0.73	0.79
	G	2.41	2.92	2.84	2.39	2.49	2.38	2.3	2.2	2.11	2.16	2.17	2.38	2.08
	γG	1.38	1.61	1.62	1.44	1.44	1.35	1.33	1.11	1.08	1.13	1.18	1.25	1.13
	NI	0.4	1.5	3.0	3.2	3.3	4.5	5.3	4.6	4.1	5.0	6.1	5.7	5.4

* Chickens reinoculated with infectious bronchitis virus.

† A/G = albumin globulin ratio; G = total globulin as grams per 100 ml. of serum; γG = *gamma* globulin as grams per 100 ml. of serum; NI = l.d.$_{50}$ neutralization index.

weeks after reinoculation on the fifteenth week of the experiment. During the subsequent four weeks to the termination of the experiment at the nineteenth week, there was a gradual decline to 5.4 (table 3, fig. 1).

DISCUSSION

Electrophoretic analyses resulted in the separation of chicken serum into five components: albumin, *alpha*-1 globulin, *alpha*-2 globulin, *beta* globulin, and *gamma* globulin. Mobilities of these components in all groups of chickens agreed essentially with the values reported by Sanders *et al.*[31] and were as follows on the basis of $cm.^2/sec./v. \times 10^{-5}$: albumin, 6.0; *alpha*-1 globulin, 5.2; *alpha*-2 globulin, 4.4; *beta* globulin, 3.6; and *gamma* globulin, 2.0.

Marked variations were encountered in the relative per cent distribution of serum components from chickens at the beginning of the experiment. The average per cent composition and range of the components were as follows: albumin, 40.2 (31.4 to 45.6); *alpha*-1 globulin, 10.1 (7.7 to 11.2); *alpha*-2 globulin, 6.7 (3.7 to 9.0); *beta* globulin, 9.5 (8.1 to 11.6); and *gamma* globulin, 33.5 (31.6 to 37.8). The average A/G was 0.67, with a range from 0.46 to 0.89. The protein content averaged 4.09 Gm./100 ml. with a range from 3.73 to 5.08 Gm./100 ml.

Sanders *et al.*,[31] using veronal buffer at pH 8.6, 0.1 ionic strength, found the following average per cent values for chicken serum components: albumin, 46.0; *alpha* globulin, 13.1; *beta* globulin, 15.7; and *gamma* globulin, 21.6. The A/G was 0.85.

Deutsch *et al.* [32] employed veronal buffer at pH 8.6, 0.1 ionic strength, and reported the following average per cent values for chicken serum components: albumin, 46.0; *alpha* globulin, 22.0; *beta* globulin, 8.0; and *gamma* globulin, 24.0. The A/G was 0.85.

In the present study, the L_1 component, as described by Sanders *et al.*,[31] was not observed in the electrophoretic patterns of chickens showing gross evidence of lymphomatosis at necropsy. It is possible that this component may be an artefact similar to that observed in the patterns from the ascending limb of the electrophoresis cell. It seems to be comparable with the *gamma* anomaly of the pattern of the descending limb. The L_1 component, or a component similar to it, was observed in the ascending patterns of serums from chickens that did not show manifestations of lymphomatosis.

The data from the experiment offer the following points for consideration: (1) the effect of the volume of blood collected at certain time intervals, (2) the effect of primary antigenic stimulus, and (3) the effect of secondary stimulus.

In the control group, it is evident that the volume of blood collected at weekly intervals for the first four weeks induced an imbalance of serum components. This is shown by the irregularity of the A/G ratios and quantitative values of the serum components until restoration of the normal equilibrium at the fourth week. The increase of albumin was probably a compensatory mechanism to maintain osmotic pressure affected by the decrease of total globulin. Osmotic pressure of albumin, owing to the greater number of particles per unit volume, is much higher than that of globulin.[33] When the same volume of blood was collected at intervals of four weeks or more, the interim was sufficient for restoration of the serum protein equilibrium.

Variations in the $l.d._{50}$ NI were encountered when blood was collected at weekly intervals as compared to the stability of the values when blood was collected at intervals of four weeks or more.

Two types of measurable response, globulin and neutralizing antibodies, resulted as a definite series of events from the primary antigenic stimulus received by groups 2 and 3. Each type was similar in both groups but there was a divergence between types. The general pattern was a rapid rise of total globulin and *gamma* globulin to a maximum within two weeks followed by a recession during the next eight weeks to levels lower than the initial level. Minor fluctuations occurred during the remainder of the experiment but none was considered to be significant.

Neutralizing antibodies showed a vigorous and continuous response with the maximum levels being attained within six to eight weeks. In group 2 which received only the primary antigenic stimulus, neutralizing antibodies gradually declined during the remainder of the experiment. The terminal value was 2.3 log units higher

than the initial value, and 1.1 log units lower than the maximum value.

The secondary antigenic stimulus received by group 3, 12 weeks after the primary stimulus, produced no significant response of total globulin or *gamma* globulin. Neutralizing antibodies, however, were increased to a level of 1.5 log units higher than the previous peak which was 4.2 log units higher than the initial level. A gradual decline occurred with the terminal value being 0.7 units lower than the maximum, but 5.0 log units higher than the initial level.

Formulation of a hypothesis as to the relation of total globulin and *gamma* globulin and neutralizing antibodies is beyond the scope of the present study. Further evidence is needed as to the residence and function of antibodies within certain serum fractions. Chemical fractionation of serum into its component parts and serological studies should be employed. In the present study, two different methods of assay were used: (1) physical assay by electrophoresis for quantitative determination of serum components, and (2) biological assay for the quantitative determination of the infectivity-neutralizing potential of serum antibodies.

It is well known that antibodies are found in globulins whose molecules are adapted complementarily to the determinant groups of the antigen. It is apparent from the present study that antibody formation was not impaired by the imbalance of serum components which occurred during the first four weeks of the experiment.[34-40] The primary antigenic stimulus was accompanied by a marked but transient increase of total globulin and *gamma* globulin. Neutralizing antibodies showed a vigorous and prolonged response. Their further presence was not dependent on the accompanying increased level of total globulin or *gamma* globulin when the infectivity-neutralizing potential of the serum was at the maximum.

SUMMARY

Electrophoretic and serum-neutralization studies were made on serums from chickens that received a primary antigenic stimulus with infectious bronchitis virus and from chickens that received, in addition, a secondary stimulus 12 weeks later.

Collection of 20 ml. of blood from the chickens at weekly intervals for the first four weeks of the experiment induced an imbalance of serum components as detected in the control group. When the same volume of blood was collected at intervals of four weeks or more, the interim was sufficient for restoration of the serum protein equilibrium.

Response to primary antigenic stimulus was not impaired by the imbalance of serum proteins. Total globulin and *gamma* globulin increased to maximums within two weeks followed by a recession during the next eight weeks to minimums which persisted for the subsequent ten weeks of the experiment. Maximum neutralizing antibody levels were attained in six to eight weeks with a slight decline during the next 12 weeks. When neutralizing antibodies were at the maximum, total globulin and *gamma* globulin were at the minimum.

Receipt of the secondary antigenic stimulus was manifested in three weeks by a further increase of neutralizing antibodies. During the next four weeks, there was a slight decline but the terminal level was above the maximum induced by the primary stimulus. No significant response of total globulin or *gamma* globulin was evident following secondary antigenic stimulus.

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The Fungous Flora of Chickens with Infections of the Respiratory Tract

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IN RECENT years, the growing economic value of the poultry industry in the United States has warranted increased research on disease problems.

The experiment of Beaudette and Hudson,[2] in 1933, on laryngotracheitis and the study of Newcastle disease (ND) by Beach,[1] in 1942, stimulated the production and the use of vaccines for the control of respiratory diseases in birds. The significance of fungous infections in birds was discussed by Witter and Chute[6] and Chute *et al.*[3] in 1952 and in 1955.

The prevalence of fungi in the lungs of poultry has been reported from England by Jordan.[7] This study was conducted on 25 birds. Species of Penicillium were found in 18 of the 25 lungs examined and *Aspergillus fumigatus* in seven; three yielded no organisms. *Aspergillus glaucus*, *Aspergillus candidus*, *Aspergillus versicolor*, Mucor sp., and an unidentified yeast were also isolated. Over a period of years, Jordan[8] also cultured 378 crops of chickens dying from a variety of causes and found *Candida albicans* in 81.

The purpose of the current study is to determine the fungous flora which might be commonly encountered in birds showing respiratory disease. The present report describes the methods used as well as the size and age of the flock, data of sampling, fungi isolated, and diagnosis of the case.

MATERIALS AND METHODS

Birds of various ages and breeds, and under different methods of management and feeding, were selected at random from the Diagnostic Service, Department of Animal Pathology, University of Maine. All birds were from Maine and all had a clinical history of respiratory disease.

After a bird was killed by separating the atlo-axoid joint, the feathers were soaked with liquid soap and the skin was removed from the ventral portions of the body. Aseptic precautions were used in removing the breast. A small portion of the abdominal or thoracic air sac, as well as the lung, was removed into a sterile jar. These tissues were taken to an inoculating room and a portion of tissue was removed aseptically and placed on plates of agar medium. Daily observations for growth were made for approximately ten days, and subcultures were made when growth of microorganisms appeared. Early in the study, tracheal tissue was removed and examined, but this practice was later abandoned because exactly reproducible results were not obtained.

Sabouraud's and Czapek's agars were the mediums used for culturing the fungous isolates although an oxgall agar was also employed in some of the early phases of the investigation.

The final diagnosis of each case was determined from gross and microscopic pathological changes. Duplicate sections of all tissues were stained by two separate techniques, one with hematoxylin-eosin, the other with Gridley's[5] fungous stain.

The serological studies were carried out using standard hemagglutination-inhibition methods for the diagnosis of Newcastle disease. In serum-neutralization tests for infectious bronchitis, 10-day-old chicken embryos and the Beaudette strain of egg-adapted infectious bronchitis virus were used throughout the study.

The diagnosis of chronic respiratory disease (CRD)[9] was based on a history of mild respiratory disease accompanied by the findings of histopathological lymphoid foci in the lungs, trachea, and air sacs. Air sac disease[10] was differentiated from CRD by (1) being much more severe, (2) usually having a cheesy exudate in the air sacs, and (3) often being complicated by pericarditis or perihepatitis.

RESULTS

The results of the study are summarized (table 1). Although *A. fumigatus* was most commonly isolated, Aspergilli belonging to the species, *Aspergillus flavus*, *A. glaucus*, and *A. candidus* were also found. Five species of Penicillium were isolated, with *Penicillium brevicompactum* being the most common. Besides Aspergillus and Penicillium, other genera of fungi isolated were Cephalosporium, Paecilomyces, Trichoderma, Scopulariopsis, and Mucor.

In the cases studied, serologically positive findings for infectious bronchitis or

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TABLE 1—Fungous Flora of Birds with a Respiratory Disease

Date	No.* sampled	Age	Fungi isolated	SN–IB**	HI–ND†	Vaccinations	Diagnosis
8-12-54	1/5,000	6 wk.	*Aspergillus fumigatus*			ND IB	Fungous infection
8-27-54	1/4,000	4 wk.	*A. fumigatus*	—	—	ND IB	Fungous infection
12-23-54	1/25,000	4 wk.	*Aspergillus flavus*, *Paecilomyces varioti*	—	1+ 3—	ND IB	Avian encephalomyelitis; upper respiratory disease
1-3-55	1/3,000	4 mo.	*Penicillium commune*, Cephalosporium sp.	+	+	ND	Air sac disease; CRD
1-13-55	3/15,300	8½ wk.	No. 1-2—*Penicillium brevicompactum*, *Aspergillus glaucus* group No. 3—*P. brevicompactum*, Mucor sp.	3+ 3—	—	IB	Air sac disease; aplastic anemia
1-13-55	2/11,000	7 wk.	No. 1-3—Negative	2 S 1—	—	ND IB	Air sac disease
1-19-55	1/25,700	8 wk.	*P. brevicompactum*, *P. varioti*	3 S 2—	1+ 4—	ND IB	Air sac disease
2-1-55	1/20,000	9 wk.	*P. brevicompactum*			ND	Air sac disease
2-2-55	2/24,000	6 wk.	No. 1-2—*P. brevicompactum*	2+ 1— 1 J		None	CRD
2-2-55	2/20,000	8 wk.	No. 1-2—Negative	3+ 2—	—	ND IB	Air sac disease
3-14-55	2/16,000	8 wk.	No. 1-2—*P. brevicompactum*	+		IB	Necrotic enteritis
3-14-55	2/11,000	12 wk.	No. 1—Trichoderma (near *T. glaucum*) No. 2—*P. brevicompactum*	+	—	ND IB	Granulomatous inflation of lung
3-14-55	4/1,200	9–10 mo.	No. 1-4—*A. fumigatus*			None	Aspergillosis
3-23-55	3/6,000	7 wk.	No. 1-3—*A. fumigatus*			None	Fungous infection
4-11-55	3/3,000	14 wk.	No. 1—*A. fumigatus* No. 2—Negative No. 3—Scopulariopsis sp.	+	2 J 1—	ND	Aplastic anemia; fungous infection
4-19-55	3/3,000	9 wk.	No. 1—*P. brevicompactum*, *Penicillium roqueforti* No. 2—*Scopulariopsis brevicaulis* No. 3—*P. roqueforti*	3— 1 J	—	IB	Aplastic anemia
4-20-55	3/10,000	8 wk.	No. 1—*A. flavus*, *A. fumigatus*, Mucor sp. No. 2—*Aspergillus candidus* (or close), *A. fumigatus* No. 3—*A. fumigatus*	—	—	ND	Pneumonia; slight hypoplasia of bone marrow
4-20-55	2/14,000	8 wk.	No. 1—*S. brevicaulis* No. 2—*Penicillium islandicum*, near *Aspergillus restrictus* (in *A. glaucus* group)	+	—	ND	
4-22-55	3/600	4 wk.	No. 1—Mucor sp. No. 2—Negative No. 3—*A. fumigatus*		—	None	Aplastic anemia
4-26-55	2/3,000	9 wk.	No. 1—*Penicillium cyclopium*, *S. brevicaulis*, *A. fumigatus* No. 2—*S. brevicaulis*	+	2 S 1+	ND IB	
4-27-55	2/5,000	3 wk.	No. 1—*Trichoderma koningi* No. 2—*S. brevicaulis*	+	—	ND	Undetermined respiratory disease
5-3-55	2/4,000	13 wk.	No. 1—*A. flavus* No. 2—*A. flavus*, *Penicillium terrestre* series	+	—	IB	CRD
5-5-55	3/8,000	7 wk.	No. 1—*P. cyclopium* No. 2—Negative No. 3—*S. brevicaulis*	+	—	None	Aplastic anemia; CRD
5-23-55	3/3,000	5 wk.	No. 1-3—*A. fumigatus*			None	Fungous infection
6-8-55	3/640	2 wk.	No. 1-3—*A. fumigatus*	3— 1 J	—	None	Fungous infection (brains, muscle, etc.)
7-1-55	2/250	3 wk.	No. 1-2—*A. fumigatus*			None	Fungous infection
7-12-55	2/16,000	11 da.	No. 1-2—*A. fumigatus* Mucor sp.			None	Fungous pneumonia
7-14-55	2/10,000	3 wk.	No. 1-2—*A. fumigatus*	2+ 1— 1 J	—		Air sac disease
7-19-55	2/10,000	1 wk.	No. 1-2—*A. fumigatus*			None	Fungous infection

* 1/5,000—1 bird of 5,000 was examined for fungi; ** serum-neutralization tests for infectious bronchitis; † hemagglutination-inhibition test for Newcastle disease; — negative; + positive; J—jelled; S—suspect.

The SN and HI columns, which show numbers tested, do not necessarily correspond to the "No. sampled" column. The reason for this is that often only a representative bird would be examined for the fungous flora; whereas, when the birds arrived at the laboratory for study, usually 4 birds were bled for SN–IB and HI–ND serology as a routine procedure.

Newcastle disease would indicate that the birds had recovered from the disease.

In view of the variety of organisms which could be isolated, previous attacks of respiratory disease, or the effects of vaccination, did not appear to materially alter the fungous flora of the lungs or the air sacs.

Discussion

The study, based on isolations of fungi from 63 birds selected from 29 flocks, which totaled 277,690 birds, gives an indication of the common types of fungi which may be present in the lungs and the air sacs. Practically all of the birds studied were of the White Plymouth Rock breed and came from large broiler plants. In view of the fact that the same organisms were isolated on a number of trials from several pieces of tissue from the same lung, it would seem that the techniques employed were reliable.

The work indicated that *A. fumigatus* was the most common fungous pathogen of the birds studied; however, tissue invasion was not established in all birds from which the organism was isolated. This points to the necessity of careful and adequate tissue examination before a positive diagnosis may be made. There is no information to show that the cultures, other than *A. fumigatus*, which were isolated were nonpathogenic.

Other studies have indicated that the fungi found growing on spilled feed or in the litter are also commonly found in the lungs. Unpublished data from our laboratory have shown that on one farm on four different occasions *Paecilomyces varioti* and *A. fumigatus* were isolated from the litter, lungs, and air sacs of birds and from the air of the poultry house.

Practically all of the cultures isolated have at some time been incriminated in domestic animal or human disease syndromes. Recently, studies by Forgacs and Carll [4] have also related "hemorrhagic syndrome" or aplastic anemia to toxic feed substrates produced by certain species of fungi growing in the feed. They reported that they produced lesions similar to those in field cases of hemorrhagic disease by feeding day-old chicks scratch grain on which selected strains of aspergilli, penicillia, and Alternaria had been cultured.

The finding of 5 completely negative birds from three different flocks would indicate that some birds, and perhaps some flocks, are relatively free from viable fungous spores or hyphae in their lungs or air sacs.

Summary

The lungs and air sacs of 63 birds from 29 flocks were cultured for fungi. The following genera were found: Aspergillus, Penicillium, Paecilomyces, Cephalosporium, Trichoderma, Scopulariopsis, and Mucor.

Aspergillus fumigatus was the pathogen most commonly encountered but it was not found invariably to invade tissue with which it was associated.

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Micropathological Changes in an Experimental Hemorrhagic Syndrome in Chickens Fed Sulfaquinoxaline and Suggested Cause of the Disease

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A CONDITION called "hemorrhagic disease" has been recognized in poultry since 1951. In some sections, it has assumed considerable economic importance.

While hemorrhages are a common feature of the disease, other lesions, including focal necrosis of the liver and spleen, anemia, pale comb and yellow bone marrow, are usually present. The exact causes of all expressions of the syndrome are not known but, in many flocks, the condition is associated with some form of medication of the feed or water.

When hemorrhagic disease first appeared, some workers associated it with vitamin K deficiency but it now appears that they are different entities.[1, 2]

Farr and Wehr[3] fed sulfamerazine to chickens and produced necrosis of the liver and spleen as well as retarded weight gains. Levine and Barber[4] found hemorrhagic infarcts in the spleens of chickens fed three different sulfa drugs. Cellular infiltration, hemorrhage and necrosis,[5] as well as necrotic, granulomatous, and inflammatory lesions,[6] were found in human patients who died after receiving sulfa drugs. Merkel[7] found focal necrosis of the liver, spleen, bone marrow, lymph nodes, lungs, and kidneys of 4 human patients who died following sulfathiazole therapy. Some of the lesions were infiltrated with polymorphonuclear cells. Young[8] reported the death of a human patient from granulocytopenia caused by sulfanilamide. The bone marrow showed complete absence of granular cells. Berg and Holtzman[9] reported a fatal case of granulocytopenia following sulfanilamide therapy.

Endicott, Kornberg, and Daft[10] produced bone marrow depression and hemorrhages in many tissues of rats fed five different sulfa drugs or acetylsulfadiazine in purified diets. Farr and Jaquette[11] found necrosis and hemorrhage in the spleens of chickens fed sulfamerazine. Delaplane and Milliff,[12] Baker and Jaquette,[13] and Davies[14] reported hemorrhages in chicks treated with sulfaquinoxaline. Gray, Snoeyenbos, and Reynolds[15] described field cases of hemorrhagic disease in which gross and microscopic lesions included hemorrhage, liver necrosis, fatty and hypoplastic bone marrow, pale blood, and intestinal ulcerations. All of the eight affected flocks had been treated with anticoccidial drugs. Cover, Mellen, and Gill[1] found hemorrhage and necrosis of the liver and intestines of chickens from field cases of the hemorrhagic syndrome. Some flocks had been treated with sulfaquinoxaline; others had never been exposed to the drug, but it was not stated whether other drugs had been used. Sadek, Hanson, and Alberts[16] found necrotic foci in the spleen, necrosis of renal tubular epitholium, watery, pale blood, pale yellowish, hypoplastic bone marrow, agranulocytosis, and muscular hemorrhage among chickens from flocks which had been treated with sulfaquinoxaline and other drugs. They observed a similarity between some of their findings and hypoplastic anemia in man.

Lodge and Woodcock,[17] Hageman and Blake,[18] Lichtenstein and Fox,[19] French,[5] More, McMillan and Duff,[6] and Heck[20] attributed the lesions following sulfonamide therapy to an allergic reaction and Davis,[21] and other authors, have demonstrated the linkage of sulfonamides to plasma protein. More, McMillan, and Duff[6] suggest that toxicity and individual idiosynerasy are not sufficient to account for all of the reactions and lesions that might follow sulfonamide therapy. Heck[20] stated that over 50 drugs are known which will produce acute reactions with agranulocytosis in man.

Moore *et al.*[22] reported hemorrhagic disease in a flock of young chickens at the Ohio Agricultural Experiment Station. The disease appeared shortly after the birds were fed sulfaquinoxaline as a treatment for coccidiosis. This incident prompted a study of sulfaquinoxaline toxicity for birds on standard rations containing adequate vitamin K and alfalfa. Yacowitz *et al.*[2] added 0.05 per cent, 0.1 per cent, and 0.15 per cent sulfaquinoxaline to the feed of ten different groups of chicks; three groups were kept as controls. All pens contained 40 or more birds. Chicks were started on the drug at different ages. Externally visible hemorrhages appeared in some chicks from all groups receiving sulfaquinoxaline, except in two in which the chicks were started on the drug at 1 day of age; however, necropsy revealed gross hemorrhages in some birds from all groups receiving sulfaquinoxaline. External hemorrhages did not appear in control birds, which received the same rations but without sulfaquinoxaline, but an

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occasional fine superficial linear hemorrhage was seen at necropsy on the legs and breasts of a few chickens. The cause of these is not known, but there was no resemblance to the extensive hemorrhagic lesions seen in the treated birds. Micropathological changes found in representative birds from this experiment are described in this paper.

Materials and Methods

Since time did not permit a detailed histopathological study of all the birds in the experiment, 2 birds were chosen at random from the control groups and 4 birds with numerous muscular hemorrhages were selected from the group which had received 0.1 per cent sulfaquinoxaline. Tissues from birds which received sulfaquinoxaline at levels of 0.05 per cent and 0.15 per cent were not studied. Heart, liver, brain, sciatic nerve, skeletal muscle, lung, kidney, spleen, thymus, proventriculus, ovary, testicle, gizzard, small intestine, pancreas, adrenal, and bone marrow from the proximal third of the tibia were saved for study. Tissues were fixed in 10 per cent neutral formalin, embedded in paraffin, and sectioned at 6 μ. All tissues were routinely stained with hematoxylin-eosin. Special stains were employed where needed.

Histopathological Findings

Treated Birds.—The bone marrow from the proximal end of the tibia of 3 treated birds was markedly hypoplastic (fig. 1). Small groups of erythrocytes were present in some sinusoids. Small lymphocytic foci could be found in which some of the cells were undergoing degeneration as indicated by karyorrhexis and pyknosis of nuclei. Where small groups of immature granulocytes remained, they were lightly stained and showed evidence of degeneration. Focal necrosis and karyorrhexis were present in the marrow of 1 bird. In some areas, the marrow had the appearance of adipose tissue but fat stains revealed a degree of fatty infiltration comparable to the marrow from the control birds.

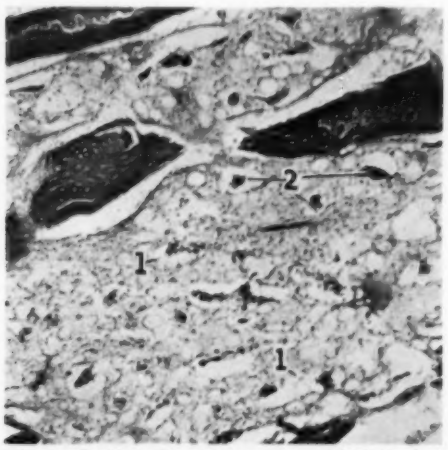

Fig. 1—Section of bone marrow, proximal third of tibia, from 7-week-old chicken treated with sulfaquinoxaline. Notice the complete absence of cells except for occasional degenerating lymphocyte shown as gray sphere with clear center (1) and erythrocytes (2) in some of the sinusoids. Individual reticuloendothial cells are scattered throughout the reticulum. Fat stain revealed only small amounts of fat. Hematoxylin and eosin. x 200.

Fig. 2—Focal myocarditis with necrosis in chicken treated with sulfaquinoxaline showing infiltration of macrophages and lymphocytes. Hematoxylin and eosin. x 320.

Neither total nor differential leukocyte counts were obtained in treated birds. Therefore, the probable presence of agranulocytosis can not be established except by inference from the bone marrow hypoplasia.

Petechial hemorrhages were present in the myocardium and skeletal muscle of 3 birds and focal myocarditis with necrosis was present in 1 bird (fig. 2). Muscle fibers had been destroyed or only remnants remained. Large macrophages and lymphocytes had invaded the lesion and infiltrated the spaces between adjacent fibers. In another area, muscle fibers had undergone hyalinization and, on cross section, appeared dense, eosinophilic, and homogenous.

Three livers showed degeneration and necrosis (fig. 3). Necrosis was more ad-

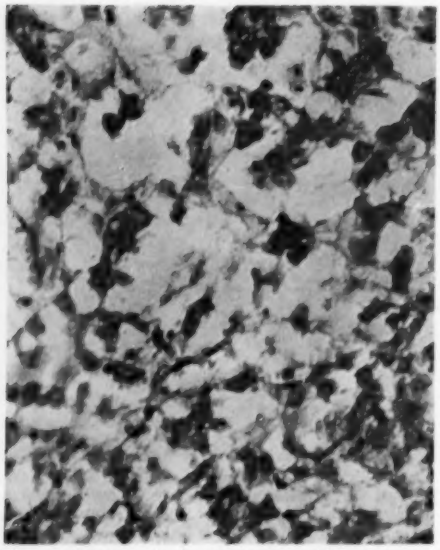

Fig. 3—Section of the liver of a chicken treated with sulfaquinoxaline showing centrolobular necrosis. The hepatic cells are represented only by distorted cytoplasmic fragments with pyknotic nuclei in some. Hematoxylin and eosin. x 440.

vanced around the central veins where some cells were completely destroyed or only distorted fragments of cytoplasm remained. Sinusoids were disrupted and large open areas were present in the tissue. Albuminous degeneration and focal necrosis could be seen toward the periphery of the lobules. Karyorrhexis was present and many hepatic cells contained vacuoles. Papillary hyperplasia of bile duct epithelium was prominent in two livers (fig. 4).

One spleen contained many areas of focal necrosis (fig. 5) which were characterized by granular, basophilic, acellular material. In some foci, large clear vacuoles could be seen suggesting liquefaction necrosis. Some of these foci were surrounded by zones of eosinophilic, hyalinized, connective tissue while others were bordered by splenic pulp.

Kidneys from 2 chickens showed moderate to severe changes in both the cortex and the medulla. These changes included albuminous degeneration of renal epithelium progressing to necrosis and complete loss of cells in some tubules. Casts had formed in some tubules and a few tubules had broken walls. Diffuse hemorrhage was present in the hilus of the kidney of 1 bird.

Lesions in the brain stems of 3 birds included neuronal degeneration in 2 and focal gliosis, neuronophagia, and petechial hemorrhages in 1 each.

The bronchial submucosa and interlobular tissue of one lung contained large clear spaces with noticeable separation of the fibrous and cellular elements indicating edema. Lymphocytes had infiltrated the edematous tissues. In one lung, thrombosis of a small blood vessel had occurred.

In the proventriculus and duodenum of 2 birds, part of the mucosa had been replaced by fibrous tissue probably as a result of an earlier acute, necrotizing enteritis. Hemorrhages were present in the lamina propria of the duodenum and proventriculus of the other 2 chickens.

Control Birds.—The bone marrow (fig. 6) was packed with cells which included erythrocytes, granulocytes, and some lymphocytes; fat stains revealed normal amounts of fat.

DISCUSSION

The control birds remained clinically healthy throughout the experiment. When they were destroyed, the blood appeared normal in color and consistency and the small hemorrhages were so infrequent as to be of no significance. The dark red bone

Fig. 4—Section of bile duct epithelium of a chicken treated with sulfaquinoxaline showing papillary hyperplasia. The edge of the lumen of the central vein is at the upper left. Hematoxylin and eosin. x 300.

marrow of untreated birds was packed with erythrocytes and mature and immature granulocytes; degenerative changes were absent in other tissues.

In contrast to the controls, treated birds which showed clinical signs of illness had pale combs and were depressed. Necropsy revealed many large and small hemorrhages in the musculature and viscera. The blood was thin and pale. Bone marrow was yellow and, microscopically, it was completely devoid of cells, in some birds, except for small groups of erythrocytes and scattered reticuloendothelial cells, while marrow from other sick birds contained small groups of cells. Other significant lesions included degenerative changes in many tissues and necrosis of the liver, spleen, and heart.

The lesions in the experimentally produced hemorrhagic syndrome closely paralleled those observed in naturally occurring field cases. They are also in accord with those described by other workers who have reported changes following the use of sulfonamides in chickens and in other animals. Also, there is a similarity between the lesions in the treated birds and those

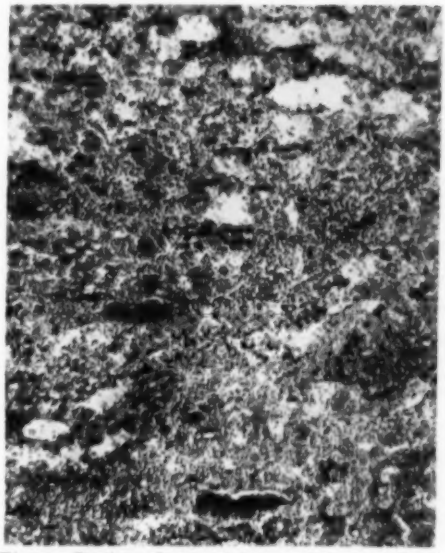

Fig. 6—Section of normal bone marrow, proximal third of tibia, from a 7-week-old untreated control chicken. The erythrocytes and granulocytes almost completely fill the spaces. Fat stain revealed only small areas of fat, which are considered normal. Hematoxylin and eosin. x 200.

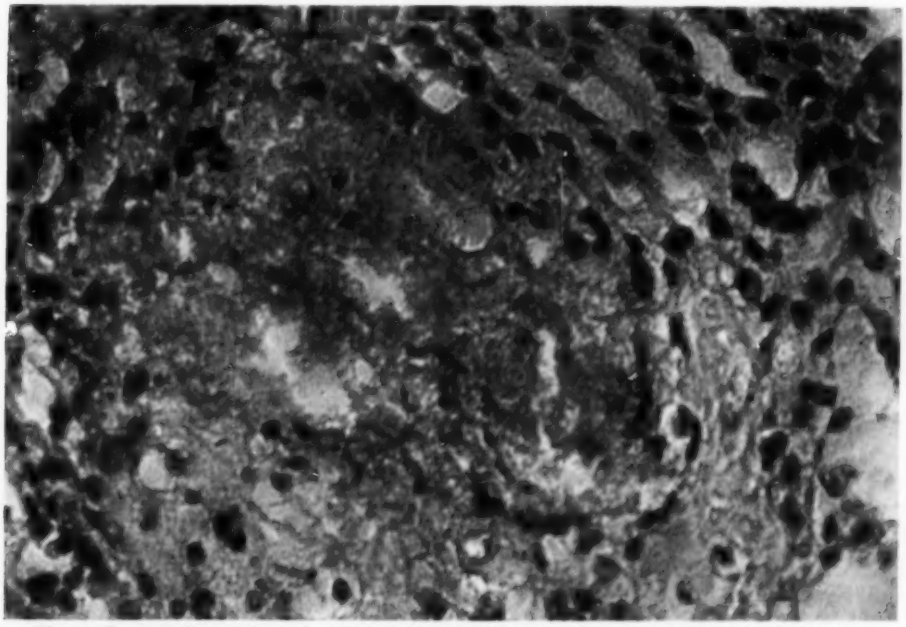

Fig. 5—Section of the spleen of a chicken treated with sulfaquinoxaline showing focal necrosis. Notice the central, granular, acellular mass with open spaces suggesting liquefaction necrosis. Hematoxylin and eosin. x 1,000.

of drug allergy following sulfonamide therapy as described in man. These observations strongly suggest that where certain drugs, especially sulfonamides, have been used, hemorrhagic disease in chickens is a manifestation of drug allergy.

Not all birds in a treated group were affected by the drug. This is in accord with reports from field cases of hemorrhagic disease. Furthermore, not all human patients treated with sulfa drugs develop a reaction. Many writers have attributed the reaction in the human patient to a drug allergy and a large number of drugs are known which will cause acute reactions in man. Since a wide variety of drugs are currently being used in the treatment of poultry diseases, some of these could conceivably produce similar reactions in chickens. It is recognized that powerful toxins can also produce a hemorrhagic syndrome.

SUMMARY

Following the addition of 0.1 per cent sulfaquinoxaline to standard chick feed, fortified with vitamin K and alfalfa leaf meal, experimental birds developed hemorrhages, focal necrosis, leukocytic infiltration, thrombosis, bone marrow hypoplasia, and anemia. Grossly visible hemorrhages were also present in birds which received 0.05 per cent and 0.15 per cent sulfaquinoxaline in the feed. The control birds remained clinically healthy and were essentially negative for lesions. A similarity of lesions was observed in the birds treated with sulfaquinoxaline in naturally occurring field cases of hemorrhagic disease in chickens and in drug allergies in man. It is suggested that many cases of hemorrhagic disease in chickens are due to drug allergy.

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Aplastic Anemia in Chickens Fed Trichloroethylene-Extracted Soybean Oil Meal and Failure of this Dietary Meal to Suppress the Development of Experimental Lymphomatosis

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NUMEROUS INSTANCES of mortality from aplastic anemia among cattle have resulted from consumption of trichloroethylene-extracted soybean oil meal (TCESOM).[7] Losses also have occurred in sheep fed this meal.[6] Although no field cases of poisoning of other species are known to have occurred, the disease has been induced experimentally in horses,[8] dogs,[9] and guinea pigs,[9] as well as in sheep[6,10] and cattle.[7] A number of other mammals, e.g., the pig,[4] rat,[9] mouse,[9] hamster,[9] and rabbit[9] are relatively resistant. No reports that poultry have been poisoned under field conditions are available. Eveleth and Goldsby,[2] however, fed chickens diets containing 22 per cent TCESOM for 50 days and produced a higher death loss, poorer rate of growth, and decreased resistance to disease as compared to controls fed hexane-extracted soybean oil meal (HEXSOM). Hemorrhages were found in the muscles and intestines of some of the TCESOM-fed chickens and the TCESOM group had a somewhat higher percentage of circulating lymphocytes than did the controls. Hill *et al.*[5] fed chickens diets containing 35 per cent, turkeys, 47.5 per cent, and geese, 37 per cent of TCESOM known to be toxic to cattle with no apparent ill-effect. In these studies, the chickens were fed throughout the period of growth and one season of egg production.

The present study was conducted to determine whether or not aplastic anemia could be induced in chickens fed semisynthetic diets containing a high percentage of TCESOM. Since TCESOM has such a profound inhibitory effect on hematopoiesis, the experiment was designed to determine, also, whether a diet high in this meal would have a suppressive effect on the development of an experimentally induced lymphomatous process.

MATERIALS AND METHODS

A total of 180 healthy, 10-day-old Rhode Island Red male chicks were separated at random into three groups of equal size and fed (table 1) the experimental diets containing (1) 72.9 per cent TCESOM known to be toxic to cattle, (2) 72.9 per cent HEXSOM obtained on the open market, and (3) a complete corn-HEXSOM diet which had long been used as the standard poultry ration by the Department of Veterinary Science.

TABLE 1—Experimental Diets Fed to Chickens

	HEXSOM	TCESOM	Regular
HEXSOM [1]	729	.....	A normal
TCESOM [2]	.....	729	complete corn-
Salt and minerals [3]	40	40	HEXSOM diet
Corn oil and vitamins A, D, and E [3]	20	20	long used by the Department
B vitamins [3]	141	141	ment of Vet-
Crisco	70	70	erinary Science
No. of chickens	60	60	60

[1] Hexane-extracted soybean oil meal obtained on the open market.

[2] Trichloroethylene-extracted soybean oil meal. This specimen induced marked aplastic anemia when fed to calves.

[3] Pritchard *et al.*[9]

At 35 days of age, one half of the chickens of each group were inoculated intramuscularly in the pectoral region with 2 ml. of a 1:10 suspension of a lymphomatous tumor—strain 12, Regional Poultry Laboratory, East Lansing, Mich. (R.P.L.–12)—obtained from a moribund chick. The agent had been passed serially ten times in chickens in our laboratory. A large dose of fully virulent agent was used in young chickens in this instance to insure the rapid development of tumors and a rapidly fatal course.

In an effort to obtain a more slowly progressing effect, 20 of the uninoculated chickens from each group were inoculated when 88 days old with 1 ml. of a 1:10 saline suspension of a lymphomatous tumor (R.P.L.–12) from the second serial passage after the agent had been stored at −40 F. for 25 months. Since none of the chickens in any of the groups developed tumors or died as a result of the latter inoculation, they were reinoculated with a fully virulent agent when they were

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TABLE 2—Characteristic Hemograms from Chickens Fed Trichloroethylene-Extracted Soybean Oil Meal (TCESOM) for 120 and 280 Days and Hexane-Extracted Soybean Oil Meal (HEXSOM) for 280 Days

Diet:	TCESOM 120			TCESOM 280			HEXSOM 280		
Days fed:									
Chicken:	5	13	28	5	13	28	82	78	85
Hematocrit (%)	19	14	11	41	43	47	42	46	49
Hemoglobin Gm./100 ml.	4.6	2.7	2.3	....	....	....	....	....	....
Erythrocytes x 10^6/mm.^{3}	1.52	0.72	0.46	3.65	3.44	3.88	3.43	2.75	3.2
Erythrocyte sedimentation rate mm. in 1 hour	2	45	30	....	....	....	....	....	....
Platelets x 10^3/mm.^{3}	7.98	2.27	0.46	29.9	22.12	27.33	49.16	29.64	53.93
Leukocytes x 10^3/mm.^{3}	11.2	14.2	3.2	21.4	23.3	22.2	30.2	26.3	34.6
Heterophils (%)	0	0	0	26	21	12	28	24	18
Lymphocytes (%)	83	61	34	70	74	81	59	72	71
Monocytes (%)	17	38	66	3	2	7	13	2	6
Basophils (%)	0	1	0	1	3	0	0	2	5
		Aplastic			Normal			Normal	

106 days old. All of the chickens which did not die were retained on the diets for a total of 280 days.

Clinical examinations were made daily and necropsies were done on all of the birds which died. Histopathological examinations were conducted on some of the chickens from all groups. Hematological examinations, including hematocrit and hemoglobin determinations, erythrocyte, platelet, and total and differential leukocyte counts, and erythrocyte sedimentation rates, were made periodically using standard methods.[5]

RESULTS

The diets were palatable and all groups made excellent weight gains except as specifically indicated. Inoculation at 35 days resulted in the development of lymphomatous tumors and death in 93, 96, and 93 per cent of those fed the TCESOM, HEXSOM, and regular diets after a rapid course that averaged 7.3, 7.4, and 8.2 days, respectively. None of the uninoculated controls developed disease.

The second inoculation was made in older chickens (88 days) with an agent that had not attained full virulence following storage and did not produce death or tumors. When the chickens were reinoculated with 2 ml. of a fully virulent agent, however, 53, 50, and 55 per cent of those fed TCESOM, HEXSOM, and regular diets developed characteristic signs and le-

Fig. 1—Note the marked repression of secondary sexual characteristics of the male fed trichloroethylene-extracted soybean oil meal on the left as compared to the control fed hexane-extracted soybean oil meal on the right.

sions of lymphomatosis and died after an average course of 16.6, 10.3, and 13.7 days, respectively. The length of the clinical course was therefore about twice as long as that following inoculation at 35 days of age. The uninoculated controls remained free of this disease.

The survivors from the inoculated groups and the uninoculated controls were continued on the experimental diets for a total of 280 days.

Beginning about the tenth week, most of the chickens fed TCESOM in both the inoculated and uninoculated groups developed characteristic signs and blood changes of aplastic anemia; 46 per cent died and showed characteristic lesions. Some of the severely anemic chickens developed lymphomatosis following the last inoculation and actually died from that disease rather than from aplastic anemia.

The most prominent clinical signs were weakness, unthriftiness, poor growth rates, pale combs and wattles, anemic and icteric mucous membranes, and diarrhea. The feces frequently contained blood. Marked depression of secondary sexual characteristics was apparent in all of the chickens fed TCESOM (fig. 1). These chickens developed blood changes characteristic of aplastic anemia. Beginning when around 18 weeks old, remission of blood changes occurred in the nonfatal cases. Characteristic hemograms for chickens with aplastic anemia, and the same chickens after remission, are indicated (table 2).

Necropsy revealed hemorrhages throughout the carcass. They were petechiae,

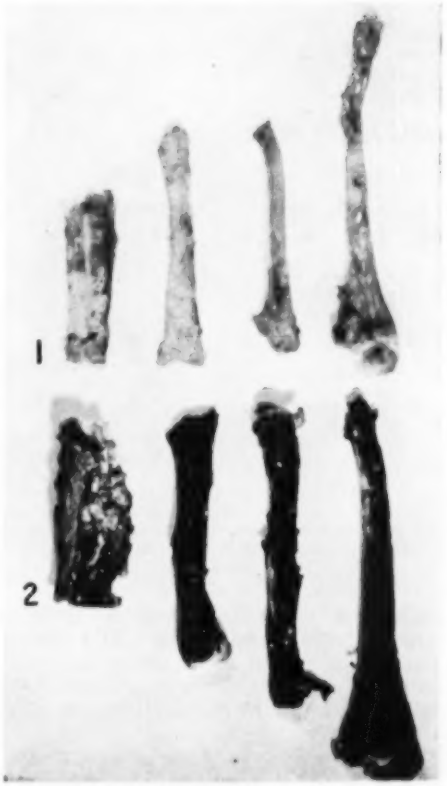

Fig. 3—Longitudinal sections of (left to right) the lumbar vertebra, humerus, femur, and tibia showing complete aplasia in a chicken fed trichloroethylene-extracted soybean oil meal, on the top (1). Those on the bottom (2) are from a control fed hexane-extracted soybean oil meal.

Fig. 2—Characteristic hemorrhages in a fatal case of aplastic anemia in a chicken fed trichloroethylene-extracted soybean oil meal.

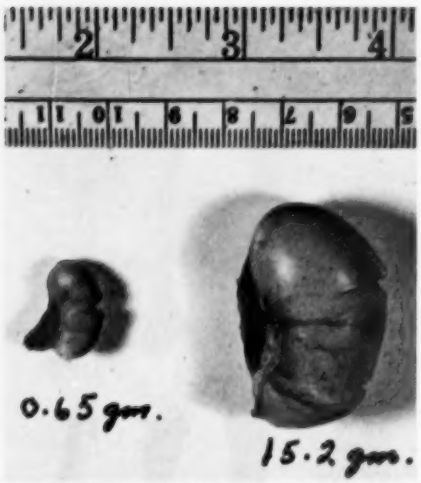

Fig. 4—Characteristic atrophic testicle (left) from chicken fed trichloroethylene-extracted soybean oil meal and normal testicle (right) from a hexane-extracted soybean oil meal-fed control, both killed at 280 days.

ecchymoses, or suffusions of blood and were most prominent on leg and pectoral muscles (fig. 2) and on the serosal and mucosal surfaces. Large accumulations of blood were found in the subcutaneous and intramuscular tissues of some. In the most anemic birds, straw-colored peritoneal, pericardial, and pleural effusions were present. Paleness of the bone marrow was the most striking finding. In some instances, the entire bone marrow cavity was filled with fat and connective tissue and no gross or histological evidence of hematopoietic tissue could be found (fig. 3). Miliary necrotic foci were present in the livers of a few birds. Gross and microscopic evidence of atrophy of the testicles was uniformly found.

Beginning at 18 to 20 weeks, the chickens that did not develop fatal aplastic anemia began to show signs of clinical improvement. They were essentially normal within six weeks, although they continued to consume the same ration of TCESOM during the entire 280-day experimental period. The only abnormal clinical finding was continued marked depression of all secondary sexual characteristics. Necropsy at the end of the study revealed atrophic testicles in the TCESOM group (fig. 4). Individual testes from this group weighed 0.65 to 1.75 Gm., while those from the HEXSOM group weighed 7.5 to 17.2 Gm.

This latter group did not show any significant histopathological changes in the

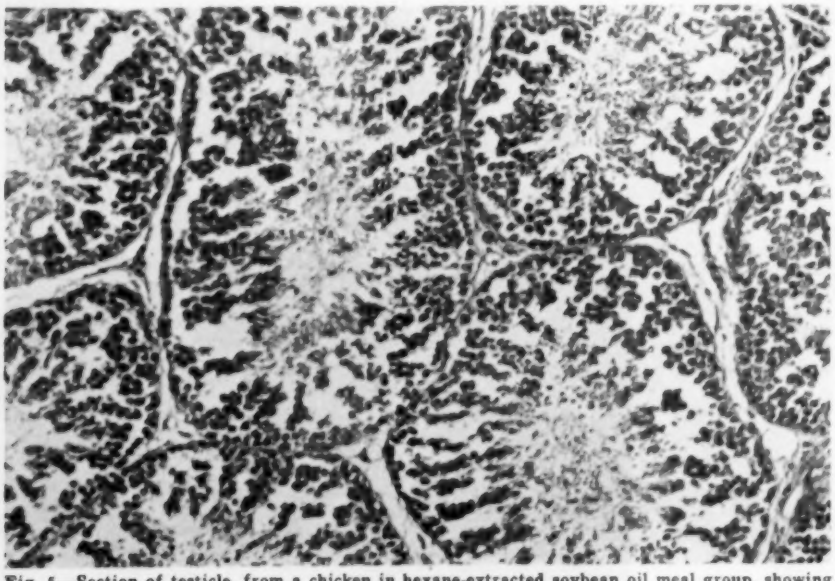

Fig. 5—Section of testicle, from a chicken in hexane-extracted soybean oil meal group, showing essentially normal seminiferous tubules. x 150.

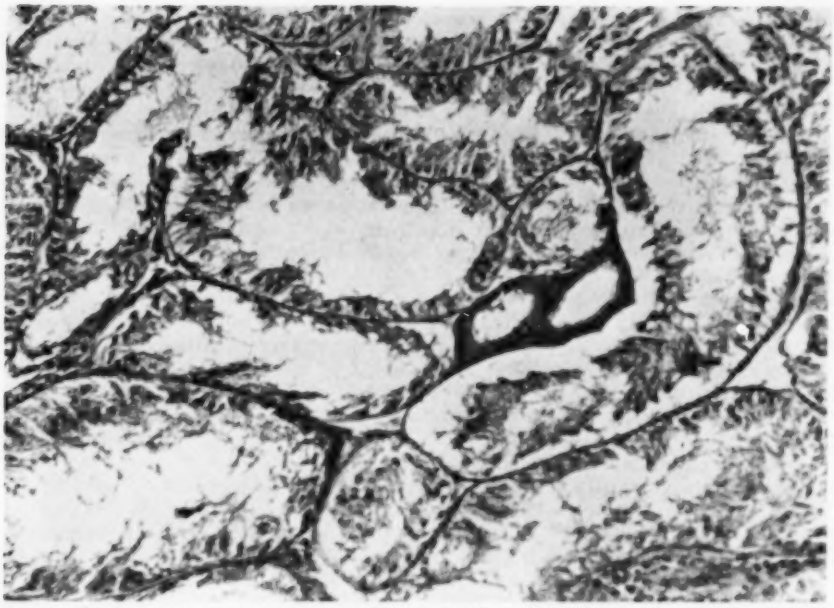

Fig. 6—Section of testicle, from a chicken in trichloroethylene-extracted soybean oil meal group, showing atrophy of seminiferous tubules and degeneration of germinal elements. x 150.

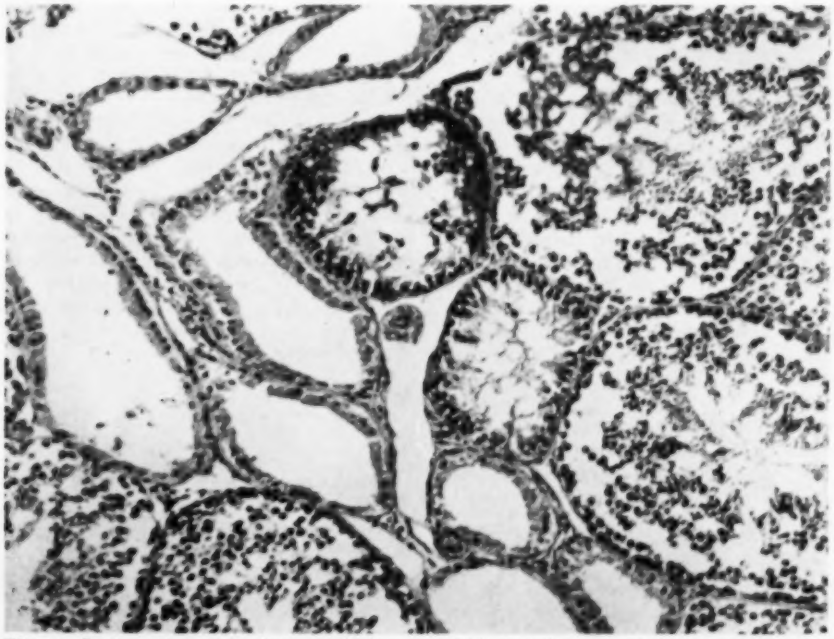

Fig. 7—Section of testicle, from a chicken in trichloroethylene-extracted soybean oil meal group, showing metaplasia of germinal elements in some tubules. x 150.

testes (fig. 5). Microscopically, the TCESOM group showed blastophthoria and metaplasia as indicated by degenerative changes and aberration of the cells within the seminiferous tubules. The tubules were smaller than normal and many were misshapen. There was relatively more stroma than normal; and there was apparently an increase of interstitial cells. The interstitial cells showed suggestions of damage in some areas—they appeared shrunken and had pyknotic nuclei.

The cells within the tubules showed marked changes in most cases. In some sections, there were areas with some stages of spermatogenetic development, with few normal spermatozoa being formed. The spermatogonial layer was usually present but it showed marked aberration. In many tubules, an aberrant layer next to the basement membrane was all that was present (fig. 6). In some tubules, there was complete loss of cells down to the basement membrane and the cells of the membrane showed beginning proliferation. The degree of aberration varied from a fringe-like zone along the luminal border to metaplasia, in which the cells were cuboidal or columnar in type (fig. 7).

DISCUSSION

The consumption of a semisynthetic diet containing 72.9 per cent TCESOM known to be capable of causing a marked inhibitory effect on hematopoiesis in cattle did not affect the development of experimental lymphomatosis in chickens inoculated at 35 and 106 days of age. This lack of effect was evident even though hematopoiesis was clearly depressed by the TCESOM at the time (106 days of age) they were inoculated, as indicated by the fact that many birds had blood changes characteristic of aplastic anemia. These results suggest that either the agent in TCESOM that depresses hematopoiesis is incapable of inhibiting the lymphomatous process or that it was present in the diet in too small a concentration to materially affect the rapidly progressing process induced by the experimental methods employed.

This study confirms the observations of Eveleth and Goldsby [2] that TCESOM is toxic to chickens and is in accord with the observations of Hill *et al.*[5] that chickens are more resistant than cattle to the toxic effects of TCESOM. The only sign of toxicity observed in chickens in this experiment was the production of aplastic anemia. The experimentally induced aplastic anemia was indistinguishable from field cases of so-called "hemorrhagic syndrome" in chickens as reported by Gray *et al.*[3] and also observed in our laboratory.[11] It is clear, however, that since TCESOM is no longer commercially available the presently occurring field cases of hemorrhagic syndrome can not be due to consumption of TCESOM.

The remission of signs and blood changes that occurred at 18 weeks could not be explained although similar remission has been observed in field cases of aplastic anemia. The marked testicular atrophy and repression of secondary male sexual characteristics was a constant finding. These changes were similar to those described by Wagener and Krüger[12] in experimentally induced hyperkeratosis in young male chickens. This suggests the possibility that the causative factors in hemorrhagic syndrome and hyperkeratosis may be similar.

SUMMARY

The consumption of a diet containing 72.9 per cent trichloroethylene-extracted soybean oil meal (TCESOM) had no noticeable effect on the development of experimental lymphomatosis in chickens. Chickens fed this diet, however, developed aplastic anemia with clinical signs and hematological and pathological changes that were indistinguishable from those seen in field cases of aplastic anemia or so-called "hemorrhagic syndrome." In addition to the pathological changes associated with hemorrhagic diathesis, these chickens developed atrophy of the testes and marked depression of secondary male sexual characteristics.

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A New Serological Technique—Freeze Agglutination
I. Application to Chronic Respiratory Disease of Chickens

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ONE of the more important problems confronting research workers in the field of so-called chronic respiratory disease (CRD) of chickens is that of developing methods of detecting antibodies produced in response to the pleuropneumonia-like organism (PPLO) associated with this disease. During the course of our investigation of this disease, a new serological technique, freeze agglutination (FA), was developed. The purpose of this paper is to describe and evaluate this technique.

Jacobs *et al.*[1] reported that of the serological tests described for the diagnosis of CRD the hemagglutination-inhibition (HI) test appeared to be the most sensitive and that the HI and the macroscopic tube-agglutination tests are best suited for thorough evaluation of the degree of infection. Since the HI and tube tests have been used most extensively, we have chosen these two as comparative tests to be run in conjunction with the FA test.

MATERIALS AND METHODS

The HI and the macroscopic tube-agglutination tests were conducted according to the method described by Jacobs *et al.*[1] Prior to use, the serums used in the HI and in the FA tests were heated for 45 minutes at 56 C. The serums used in the macroscopic tube-agglutination test were not inactivated by heat.

The antigen, the Van Roekel PPLO strain, used in the FA test was propagated in Difco PPLO medium without crystal-violet. One in 2,000 parts thallium acetate, 1,000 units penicillin per milliliter, and 1 per cent Difco PPLO serum fraction were added to the medium. After seven to eight days, the PPLO reached maximum growth. The culture was then centrifuged from the medium at 13,000 times gravity and resuspended in HI buffer* to obtain an optical density of 35 (HA activity of 1/64).

The FA test was run in disposable tubes† made from 4.1-mm. O.D. glass tubing cut into 55.0-mm. lengths and sealed at one end. The sealed ends of the tubes were inserted a distance of 0.5 cm. into a rack which held the tubes snugly in a horizontal position.

The technique of the test is as follows: Serum is delivered into the tubes to a depth of 1.0 to 2.0 cm. from a Pasteur pipette allowing an air space of 1.0 cm. to remain at the bottom of the tube. The serum is frozen in the tubes in a subzero unit or a CO_2 ice box. The rapid freezing prevents the subsequently added antigen from falling out of suspension. After freezing, the tubes are removed from the freezer and an equal amount of antigen is delivered into the tubes adjacent to the serum. The tubes are incubated in a horizontal position for five minutes at 37 C., during which time the antigen layers above the thawed serum. The liquid does not flow from the tubes because of their small inside diameter. The layered serum and antigen are frozen in a horizontal position and then reincubated at 37 C. until thawed. The tubes are subsequently slowly rotated to the vertical, slowly inverted, rotated to an upright position again, and read.

All birds used in the experiment were from 3 to 6 weeks of age. All birds were inoculated with 0.2 ml. of egg-propagated PPLO (W strain, obtained from affected chickens from Winchester, Va.) via the left posterior abdominal air sac. All birds were fasted overnight prior to the withdrawal of blood samples.

The first group of birds (table 1) was 3 weeks of age when inoculated. Samples of blood were taken from members of this group at three through 12 days after inoculation. The second group of birds (table 2) was 3 weeks of age when inoculated. Pairs of blood samples were taken from members of this group two through 16 days after inoculation. The third group of birds (table 3) was 5 weeks of age when inoculated. Blood samples were taken from 13 of these birds at five and again at 14 days after inoculation and from 9 birds at nine and again at 14 days after inoculation.

RESULTS

In this experiment, tube-agglutination

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* The stock solution consists of sodium chloride, 170.0 Gm.; potassium dihydrogen-phosphate, 13.6 Gm.; sodium hydroxide, 3.0 Gm.; and distilled water to make 1,000 cc. Fifty cubic centimeters of stock solution diluted to 1,000 cc. with distilled water gives a buffered saline having a pH of 7.1 to 7.2 and a molarity of 0.005 (Eagle, H.: Diagnosis of Syphilis. Mosby and Co., St. Louis, 1938).

† Supplied by Wheaton Glass Co., Millville, N. J.

TABLE 1 — Comparative Results of Serological Tests for PPLO Antibodies

Band No.	No. of days post-inoculation	FA*	HI†	Tube test‡
1	3	—	8	—
2	4	—	8	—
3	4	—	8	—
4	4	—	8	—
5	5	—	8	—
6	5	—	8	—
7	5	—	8	—
8	7	+	8	—
9	7	+	8	—
10	7	+	8	—
11	8	++	16	—
12	8	++	16	1:5
13	8	++	16	—
14	9	++	16	1:10
15	9	++	32	1:10
16	9	++	32	1:40
17	10	+++	32	1:10
18	10	+++	32	1:10
19	11	++++	64	1:40
20	11	++++	64	1:20
21	12	++++	32	1:20
22	12	++++	32	1:20

At 3 weeks of age, all birds were inoculated with 0.2 ml. of egg-propagated PPLO via the left posterior abdominal air sac.

* Degree of serum reactivity based on size of clumped material (fig. 1).

† *Beta* procedure, based on serum dilution.

‡ Dilution of serum required to produce complete agglutination of antigen.

titers of 1:5 and greater, and HI titers of 1:16 and greater, are regarded as indicative of the presence of PPLO antibodies.

When reading the results of the F-A test, serums producing no clumped material are regarded as negative. For qualitative estimates of titer, serums producing clumps are regarded as positive for PPLO. For rough quantitative estimates of antibody titer, serums producing clumped material averaging 2.0 mm. in diameter are regarded as 4+; 1.0 mm., 3+; 0.5 mm., 2+; and smaller clumps as 1+ (fig. 1). Serums from normal birds; birds inoculated with PPLO for fewer than five days; birds affected with Newcastle disease, leukosis, pullorum disease, paratyphoid, infectious bronchitis; or from birds infected with *Escherichia coli* react negatively to the test. Serums from birds infected with PPLO react positively to the test.

The comparative results obtained from testing the serums of 74 PPLO-inoculated birds are shown (tables 1, 2, and 3). These results, in conjunction with comparative observations made while setting up the three tests, indicate that the HI and the macroscopic tube tests for PPLO require the use of more time and glassware than does the FA. The FA test is sensitive enough but not overly sensitive and apparently permits an estimation of antibody titer.

Discussion

Serums frozen while conducting FA tests appear to separate into many clear and into many amber-colored fractions. When the serums are subsequently thawed, the amber fractions thaw before the clear fractions. This difference in melting points indicates that the amber fractions contain more salts and (or) proteinaceous material than do the clear fractions. Since *gamma* globulin is proteinaceous in nature, it seems possible that the amber fractions

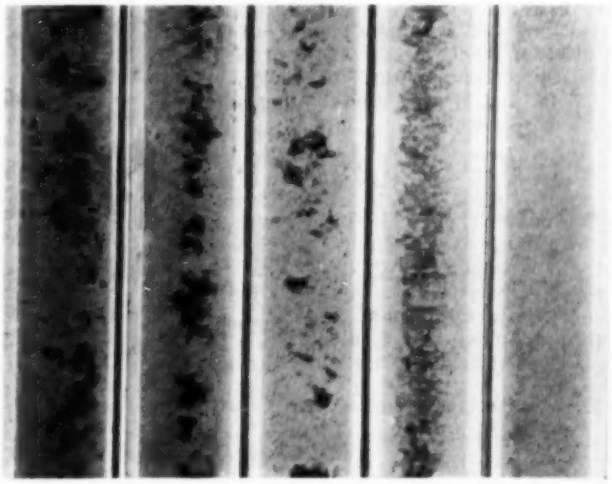

Fig. 1 — Freeze-agglutination reactions.

Left to right:

Tube containing clumped material averaging 2.0 mm. in diameter is regarded as 4+; 1.0 mm., 3+; 0.5 mm., 2+; and smaller, 1+.

Tube containing no clumped material is negative.

The clarity of the fluid mixture in which the clumped material is suspended is directly proportional to the size of the clumps.

TABLE 2 — Comparative Results of Serological Tests for PPLO Antibodies

Band No.	Days post-inoculation	FA*	HI†
23	2	—	8
24	2	—	8
25	3	—	8
26	3	—	8
27	4	—	8
28	4	—	8
29	5	1+	16
30	5	—	16
31	6	2+	8
32	6	—	8
33	7	4+	16
34	7	4+	16
35	8	3+	16
36	8	3+	16
37	9	4+	32
38	9	4+	32
39	10	4+	32
40	10	4+	16
41	11	4+	32
42	11	4+	16
43	12	4+	32
44	12	4+	32
45	13	4+	32
46	13	4+	32
47	14	3+	16
48	14	—	4
49	15	4+	32
50	15	4+	32
51	16	4+	64
52	16	4+	64

At 3 weeks of age, all birds were inoculated with 0.2 ml. of egg-propagated PPLO via the left posterior abdominal air sac.

* Degree of serum reactivity based on size of clumped material (fig. 1).

† *Beta* procedure, based on serum dilution.

TABLE 3 — Comparative Results of Serological Tests for PPLO Antibodies

Band No.	FA*	HI†	Band No.	FA*	HI†
5 days after infection			*14 days after infection*		
53	—	16	53	4+	64
54	—	4	54	4+	64
55	—	8	55	4+	32
56	—	16	56	4+	32
57	—	4	57	4+	128
58	—	4	58	4+	64
59	—	8	59	4+	128
60	—	8	60	4+	32
61	—	2	61	4+	64
62	—	8	62	4+	64
63	—	8	63	4+	32
64	—	4	64	4+	64
65	—	8	65	3+	32
9 days after infection			*14 days after infection*		
66	—	4	66	3+	64
67	3+	32	67	3+	64
68	—	8	68	4+	64
69	3+	32	69	4+	64
70	1+	16	70	3+	16
71	2+	16	71	4+	32
72	—	8	72	4+	32
73	1+	8	73	3+	32
74	—	8	74	3+	64

At 5 weeks of age, all birds were inoculated with 0.2 ml. of egg-propagated PPLO via the left posterior abdominal air sac.

* Degree of serum reactivity based on size of clumped material (fig. 1).

† *Beta* procedure, based on serum dilution.

contain the *gamma* globulin fraction of serum. Similarly, it also seems possible that freezing could concentrate the PPLO antigen. Thus, it appears plausible that the FA test could be described as a method of concentrating antigen and its specific antibody to facilitate their union. Additional work which we have done has shown that the test will also detect antibodies produced in field cases of CRD of chickens and infectious sinusitis of turkeys, and that the test is applicable to other antigen-antibody systems.

SUMMARY

A new serological technique, freeze agglutination (FA), has been developed to detect birds infected with pleuropneumonia-like organisms (PPLO). The test is conducted as follows: Serum is delivered into capillary tubes and frozen. A PPLO antigen, grown in a modified Difco PPLO medium and concentrated, is subsequently delivered into the tubes adjacent to the serum and the tubes are incubated horizontally for 5 minutes at 37 C. The layered serum and antigen are then frozen horizontally, thawed in a horizontal position at 37 C., rotated to the vertical, slowly inverted, rotated to an upright position, and examined for the presence or absence of clumping. Serums producing no clumped material are regarded as negative and serums producing clumped material are regarded as positive for PPLO.

Data obtained as a result of comparing the FA test with the HI and the macroscopic tube-agglutination test indicate that, under the conditions of this investigation, the FA test is relatively specific, sensitive, and comparatively economical to run.

Preliminary evidence indicates that suitable modifications of the test will also be useful in other antigen-antibody complexes.

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The Recovery of Rats Administered Diisopropyl Phosphorofluoridate as Influenced by Female Sex Hormones, Blood Injections, Vitamin B_{12}, and Iodinated Casein

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RELATIVELY little is known concerning the relationship between the acetylcholine esterase, found in red blood cells and nervous tissue, and the female sex hormones.

Low brain cholinesterase activity has been found in dogs induced into estrus by folliculin.[1] The brain cholinesterase activity, as well as the activity of the nonspecific cholinesterase in the blood serum, liver, and muscle of rats, was markedly increased during hyperestrus induced by large amounts of estradiol.[2] The serum cholinesterase activity of mature female rats was higher than in mature males, or in immature or senile females.[3] Ovariectomy resulted in a fall in serum cholinesterase activity which was reversed by estradiol injections but not by progesterone.[4] The serum cholinesterase activity was low during periods of low estrogen levels, such as the first half of pregnancy, pseudopregnancy, and lactation, while it was high during periods of high estrogen levels, such as estrus and the last half of pregnancy.[5] The liver cholinesterase activity of female rats was lowered by ovariectomy and was returned to normal by estradiol injections, but not by progesterone.[6, 7]

Blood injections might affect cholinesterase activities of animals poisoned with diisopropyl phosphorofluoridate (DFP) and be a supportive therapy since, beside other attributes in treating shock, the injected blood would be a source of homologous cholinesterase. Some benefit has been reported in treating dogs in traumatic shock with large doses of horse serum cholinesterase.[8] Mendel and Hawkins[9] observed that the injection of various heterologous cholinesterases gave rats a short-lived protection from the effects of injected acetylcholine and also caused pupil dilation. Protection from injected acetylcholine was also found in dogs and cats given heterologous cholinesterase.[10] In contrast, Beck[11] found no protection from injected acetylcholine in rats given large doses of bovine erythrocyte cholinesterase and, furthermore, he[12] found no alteration or reduction in mortality in DFP-poisoned rats given this heterologous cholinesterase. Mazur and Bodansky[13] found no effects on the mortality or signs of poisoning when large doses of various heterologous cholinesterases were given rabbits and monkeys poisoned with DFP.

Since diseases affecting hematopoiesis have been shown to influence red cell cholinesterase activity,[14, 15] vitamin B_{12} might affect red cell cholinesterase activity in rats poisoned with DFP.

While much is known on the relationship between thyroid hormone and nonspecific cholinesterase, little is known of the relationship with acetylcholine esterase. In dogs rendered distinctly hyperthyroid by thyroid feeding, the ratio of acetylcholine to cholinesterase activity in the brain was increased.[16] The serum cholinesterase activity of rats was reduced by thyroxine administration, while it was increased by thyroidectomy or thiouracil feeding, but the cerebral cholinesterase activity was the same in the treated and the untreated groups of both sexes.[17]

If female sex hormones, blood injections, vitamin B_{12}, or iodinated casein * influence the recovery of rats poisoned with DFP, an anticholinesterase compound, applications might have value in therapy. Iodinated casein was used because it is easily produced and contains an assayable amount of thyroxine. A correlation between red cell and brain cholinesterase activities has been demonstrated in rats poisoned with DFP[18] and, thus, periodic determinations of red cell cholinesterase activity would be expected to be an indication of the recovery of brain cholinesterase activity.

METHODS

Female Wistar rats were used in an environmental temperature controlled at 75 F., with the relative humidity at 45 per cent. A commercial rat diet was fed *ad libitum*. Blood specimens were obtained weekly by cardiac puncture while the rats were lightly etherized, except that no specimens were obtained for one week after the administration of DFP, since this procedure would cause excessive losses at that time. In the first trial, red cell cholinesterase activity was determined by the method of Marchand,[19] while in the second trial the method of Michel[20] was used. Brain specimens were obtained immediately after exsanguination by severing the aorta without anesthesia, and cholinesterase activity was determined by a modification of the constant pH method of Glick.[21] The following solutions were placed in a 10-ml. beaker equipped with a constant-stirring apparatus and maintained at 26 C.: 0.2 ml. of 10 per cent entire brain homogenate in 0.6 M KCl, 1.0 ml. veronal

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* Iodinated casein used was a product of Cerophyl Laboratories, Kansas City, Mo., and is called "protamone."

buffer (0.004 M veronal at pH 7.4), and 2.8 ml. 0.6 M KCl. The resulting mixture was adjusted with a Beckman pH meter to slightly above pH 7.4 by adding either dilute NaOH or HCl solutions. Then, 1.0 ml. of 0.11 M acetylcholine chloride in 0.6 M KCl solution was added and the time noted when the pH was reduced to 7.4. Thereafter, the pH was maintained at 7.4 by the addition of small amounts of 0.01 N NaOH with the time intervals taken. The activity was expressed in the number of milliequivalents of hydrogen ions times 10^{-5} produced per minute by 20 mg. of wet tissue.

TABLE 1—Mortalities of Female Rats Administered Diisopropyl Phosphorofluoridate and Atropine

Group	Mortality
FIRST TRIAL, 2.25 MG./KG. DFP TO 10-11-MONTH-OLD RATS	
Ovariectomized	4/10
Ovariectomized receiving estradiol	4/10
Ovariectomized receiving progesterone	2/9
Intact control	3/10
SECOND TRIAL, 1.75 MG./KG. DFP TO 5-MONTH-OLD RATS	
Blood injection	4/12
Vitamin B_{12}	4/12
Iodinated casein	3/12
Control	8/12
THIRD TRIAL, AS IN SECOND TRIAL	
Blood injection	9/56
Iodinated casein	5/56
Control	4/56

Mortalities were recorded for 72 hours after DFP exposure. In the second trial, the mortality in the control group was significantly greater ($p < .05$) than the other groups in the trial.

Numerator — number died; denominator — number in group.

In the first trial, rats weighing 250 to 325 Gm. and 10 to 11 months old were separated into four groups of 9 to 10 each by separating litters and attempting to equalize the mean normal red cell cholinesterase activity between groups. The mean normal values were calculated from two weekly samples obtained shortly before treatment. Ovariectomies were performed two months before trial. Simultaneously, rats were injected subcutaneously with 2.25 mg. per kilogram of body weight of DFP in peanut oil and 1 mg. per rat of atropine sulfate in saline. The groups were intact females; ovariectomized females; ovariectomized females receiving subcutaneous injections of 0.132 mg. α-estradiol benzoate in oil three times a week; and ovariectomized females receiving subcutaneous injections of 0.550 mg. of progesterone in oil three times a week. The hormone injections were started immediately after DFP exposure.

In the second trial, 5-month-old rats weighing 175 to 225 Gm. were separated into four groups of 12 in the same manner as the first trial. Exposure to DFP and atropine administration were the same as in the first trial, except that 1.75 mg. per kilogram of body weight of DFP was given. The groups were the controls; females receiving 10 ml. per rat of fresh heparinized rat blood (drawn from normal rats less than three hours before) intraperitoneally 30 minutes after exposure and 5 ml. the next day; females receiving daily subcutaneous injections of 10 μg. of vitamin B_{12} from the time of exposure; and females receiving oral doses of 4 mg. per rat of iodinated casein the day of exposure and the next day, but, thereafter, the iodinated casein was added to the feed at the rate of 0.02 per cent.

In the third trial, 168 rats of the same description as those used in the second trial were placed at random in 24 cages, which in turn were placed in three groups with a few additional cages of rats held in reserve to replace fatalities. After one cage of rats from each group was used to determine pre-exposure brain cholinesterase activity, the remaining rats were given DFP and atropine as in the second trial. The groups were controls; females receiving blood injections; and females receiving iodinated casein, the latter two groups receiving the treatments as described in the second trial. Brain cholinesterase activity was determined on one cage of rats per group at one day and then at weekly intervals after DFP exposure.

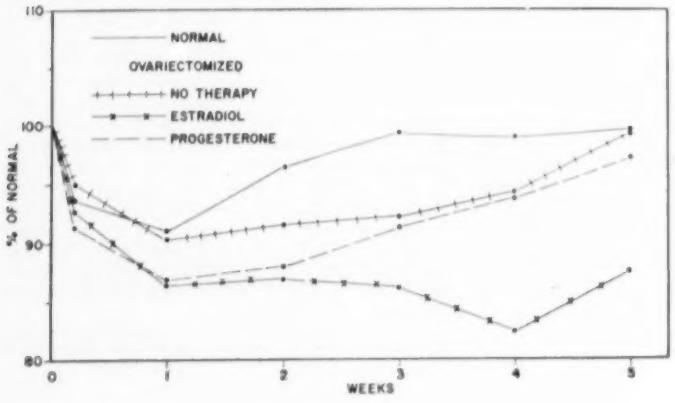

Fig. 1—The recovery of body weight in female rats administered diisopropyl phosphorofluoridate in the first trial.

RESULTS

No plausible interpretation could be advanced for the variation in the mortality to DFP seen between trials (table 1). The signs of poisoning included chromodacryorrhea * for three days, muscular

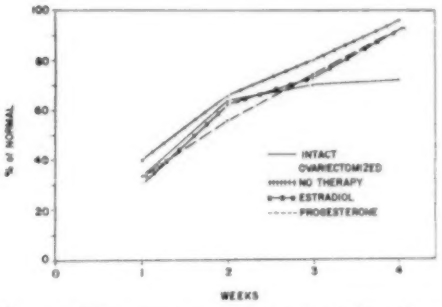

Fig. 2—The recovery of red cell cholinesterase activity in female rats administered diisopropyl phosphorofluoridate in the first trial.

fasciculation for one to two days, general weakness for five days, difficulty in grasping food for seven to ten days, and an increased sensitivity to ether for one to two weeks. In the first trial, ovariectomized rats receiving estradiol recovered from these infirmities most rapidly but also failed to recover pre-exposure weight (fig. 1). This latter finding was expected since rats in estrus tend to lose weight. In the second and third trials, rats receiving blood injections appeared to have undergone less shock and recovered most rapidly.

The average normal red cell cholinesterase activity for 48 female rats in the second trial was 0.76 ± .09 △ pH, and this value could not be differentiated statistically from 0.73 ± .09 △ pH found for 33 normal male rats in a preliminary study. The recovery of red cell cholinesterase activity was about the same in the first and second trials, all groups averaging 17 ± 2 per cent per week in the first trial (fig. 2) and 15 ± 2 per cent per week in the second trial (fig. 3). The pattern of recovery of red cell cholinesterase activity of the group receiving blood injections in the second trial differed from the other groups in that values were high one week after injection of DFP but low three weeks after injection. Perhaps, at first the injected erythrocytes functioned in some manner to increase the red cell cholinesterase activity but, later, may have depressed hematopoiesis. The injected blood was probably absorbed intact from the peritoneal cavity since necropsy within one day of death failed to account for the amount of blood injected, and the hematocrit of the survivors was 60 to 70 per cent one week after injection. The hematocrit of normal colony rats was usually 40 to 50 per cent.

In the third trial, the average pre-exposure brain cholinesterase activity for 21 normal female rats, as determined by the constant pH method, was 12.19 ± 1.93 meq. of hydrogen ions times 10^{-5} produced per minute by 20 mg. of wet tissue. The recovery of brain cholinesterase activity for all groups averaged 17.6 ± 2.6 per cent per week from one day through three weeks after injection of DFP, but no further recovery was apparent from three through six weeks (fig. 4). The range for all groups, three through six weeks after DFP, was 58.2 to 81.7 per cent of normal, with the group receiving iodinated casein tending to have values below other groups at three, five, and six weeks after DFP injections. Rats succumbing to the poison were not included in the tabulations, but it was of interest that when the brain cholinesterase activity was determined a short time after death, the activity was at, or very near to, zero.

Data were analyzed for significance ($p < .05$) with each rat serving as its own con-

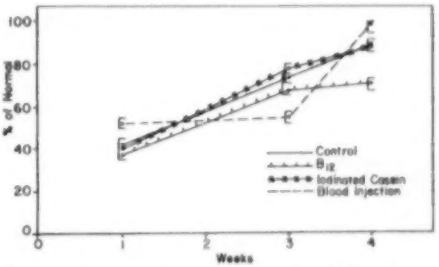

Fig. 3—The recovery of red cell cholinesterase activity in female rats administered diisopropyl phosphorofluoridate in the second trial. Values at two weeks were not included because the apparatus was not working. The brackets note significant differences ($p. < .05$). The low value for the vitamin B_{12} group at four weeks may have been an artifact because of deaths within the group.

* Chromodacryorrhea refers to the production of tears containing porphyrins and producing reddish crusts about the eyes.

trol in the determinations of body weight and red cell cholinesterase activity. The unexposed rats were considered as normal in the determinations of brain cholinesterase activity.[22]

DISCUSSION

The recovery of red cell cholinesterase activity did not appear to be influenced by the amount of DFP given, since 2.25 mg. per kilogram was given in the first trial and 1.75 mg. per kilogram was given in the second trial, and yet the recovery rates were about the same in both trials (fig. 2 and 3). Koelle and Gilman [23] also found that considerable time was required for the recovery of brain cholinesterase activity in rats poisoned with DFP (fig. 4).

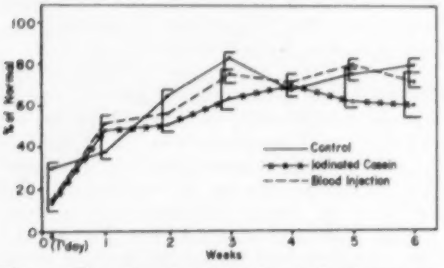

Fig. 4—The recovery of brain cholinesterase activity in female rats administered diisopropyl phosphorofluoridate in the third trial. The brackets note significant differences ($p < .05$).

There was no apparent influence of ovariectomy, estradiol, progesterone, vitamin B_{12}, or iodinated casein on the recovery of red cell cholinesterase activity. Since Lührs and Willig [2] observed an increase of various cholinesterase activities when they used a dose of estradiol which was larger than used here, a larger dose of estradiol may have influenced the recovery of red cholinesterase activity. Rats receiving blood injections showed a higher red cell cholinesterase activity than other groups one week after DFP (fig. 3), which was longer than found by others when they injected heterologous cholinesterases into various species.[9-13] Injected blood could serve many functions in this type of shock such as supplying homologous cholinesterase, nutrients, and erythrocytes. The increased number of erythrocytes might be of value, since there is probably tissue anoxia in DFP-poisoned rats. Rats showed less shock after the administration of DFP when given blood injections.

While the red cell cholinesterase activity was relatively high in DFP-poisoned rats given blood injections at one week, there was no evidence of any transfer of cholinesterase from the injected blood to the brain tissue of the recipient, since other DFP-poisoned rats also given blood injections did not have a brain cholinesterase activity that was higher than the control group at one day and one week (fig. 4). Others have not found any transfer of injected heterologous cholinesterases to the nervous tissue in several species,[10,13] although the work of Mendel and Hawkins [9] would indicate some action of injected heterologous cholinesterase upon the myoneural junction in rats.

The dosage of iodinated casein was sufficient to cause mild thyroidal stimulation,[24] and it appeared that this thyroidal stimulation retarded the recovery of brain cholinesterase activity (fig. 4) but did not influence the recovery of other body functions.

SUMMARY

The recovery of red cell cholinesterase activity in female rats poisoned with diisopropyl phosphorofluoridate (DFP) was not influenced by ovariectomy, by injections of estradiol or progesterone in ovariectomized females, by daily injections of vitamin B_{12}, or by feeding iodinated casein. Female rats poisoned with DFP and given blood injections showed an irregular pattern of recovery of red cell cholinesterase activity, the activity being higher than the controls one week after DFP injections. The normal red cell cholinesterase activity for female rats was about the same as that of males.

The recovery of brain cholinesterase activity of DFP-poisoned female rats was not influenced by blood injections, but appeared to be retarded by iodinated casein feeding. Complete recovery of brain cholinesterase activity was indicated to require considerable time.

Ovariectomized rats receiving estradiol recovered a normal appearance more rapidly than controls after DFP poisoning, and female rats receiving blood injections did not appear to have undergone as great a degree of shock as the others after being given DFP and they also recovered rapidly.

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The Susceptibility of Weaned Mice to *Escherichia Coli* and *Salmonella Typhimurium* Endotoxins During, and Subsequent to, Cortisone Treatment

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ADRENALECTOMIZED rats have an increased susceptibility to various toxins, such as streptococcic, meningococcic, and diphtheria toxins, and also to the toxic effect of typhoid vaccine.[3, 11] These toxins also seem to induce toxic adrenal insufficiency which is characterized by adrenal hemorrhage and necrosis, hypothermia, muscular weakness, and death.[7, 10]

Adrenal cortex transplants or cortical extracts increase resistance of adrenalectomized animals to the toxicity of killed *Bacillus typhosus* and *Micrococcus aureus*,[4] and 11-oxysteroids and 11-dihydrocorticosterone have been found to have a high protective action against the toxic effect of typhoid vaccine.[6] Adrenal cortical extracts and cortisone also have a beneficial effect in intact animals against diphtheria toxin [2, 23] and adrenal cortical hormone extract has been reported to have delayed death in experimental *Clostridium welchii* infection in guinea pigs.[5] Cortisone has a remarkable action on the response to endotoxins of gram-negative bacteria.[17, 19] Mature rabbits are normally vulnerable to such endotoxins, but a single injection of cortisone prior to injection of endotoxin completely eliminates the toxic effect.[20] However, young, growing rabbits are normally highly resistant to these same endotoxins but, if they are given cortisone injections prior to endotoxin injection, they develop bilateral necrosis of the kidney and necrotizing lesions of various other organs. These lesions are similar to those of the so-called "generalized Schwartzman reaction" which is also produced by gram-negative bacterial endotoxins.[1, 1', 21]

The lethal effect of Brucella endotoxin in mice is prevented when cortisone is given shortly after the endotoxin.[16] Treatment with cortisone or adrenal cortical extract has also been shown to diminish toxic signs of diseases such as typhoid, brucellosis, postoperative erysipelas, lymphangitis, and virulent ethmoid sinus infection.[14, 15, 22]

The following experiments are an attempt to evaluate and compare the effect of *Escherichia coli* and *Salmonella typhimurium* endotoxins during and subsequent to cortisone administration.

MATERIALS AND METHODS

Experimental Animals.—Weaned mice, 3 weeks old, were obtained from a colony of Webster Swiss white mice which has an extended history of freedom from infection and low death losses. The weaned mice were given "Purina lab chow" and fresh tap water, *ad libitum*.

Endotoxins.—*Escherichia coli* (0-101) and *S. typhimurium* were grown on brain-heart infusion agar (Difco) for 24 hours at 37 C., then washed off with physiological saline solution in such quantities as to give a suspension density equal to a MacFarland tube No. 8. These bacterial suspensions were heated at 80 C. for one hour and then tested for sterility by subculture on blood agar plates incubated at 37 C. for 48 hours.

The intraperitoneal average lethal dose ($l.d._{\infty}$) for weaned mice of both endotoxins was found to be approximately 0.5 ml. The heated suspensions were stored at 4 C.

Cortisone.—Saline suspension of cortone® acetate* (cortisone acetate), in a dose of 2.5 mg. per mouse per day (0.1 ml. of a suspension containing 25.0 mg./ml.), was injected intramuscularly in the thigh region.

Experimental Procedures.—Cortisone was administered daily for three successive days and then withdrawn. At various time intervals subsequent to the last cortisone injection, 20 mice were randomly selected and inoculated with endotoxin. One control group of 20 mice received no cortisone but were inoculated with endotoxin; another 20 received cortisone but no endotoxin (table 1).

RESULTS

In the control groups (table 1) which received cortisone, a few mice became slug-

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* Sharp and Dohme Division, Merck and Co., Philadelphia, Pa.

TABLE 1—Susceptibility of Weaned Mice to Endotoxins of Escherichia Coli and Salmonella Typhimurium During and Subsequent to Cortisone Administration

Type endotoxin	Control groups		Time of endotoxin administration (hours after last cortisone injection)						
	No cortisone, endotoxin	Cortisone, no endotoxin	0	12	24	36	48	72	96
E. coli (0-101)	6/20*	1/20	1/20	8/20	11/20	13/20	19/20	17/20	19/20
S. typhimurium	8/20	2/20	3/20	†	†	†	14/20	†	†

* Numerator = number dead; denominator = total number of animals in the group; † not done.

gish and developed a ruffled coat. In the zero- and 12-hour groups, few animals other than those which died showed signs of illness. Almost all animals in the other groups showed more or less marked signs of illness.

Signs of illness, when observed, occurred within seven to 12 hours after inoculation of endotoxin. The signs were a ruffled coat, a hunched-up posture, respiratory distress, and diarrhea. Many animals recovered clinically within 12 to 24 hours. Deaths usually occurred within 48 hours after injection of endotoxin.

Judging by the number of animals which died in the various groups, the results suggest an increased resistance to both endotoxins during cortisone administration and an increased susceptibility subsequent to withdrawal of cortisone.

In the case of *Esch. coli* endotoxin, maximum susceptibility seemed to be established approximately 48 hours after the last cortisone administration and continued at least 48 hours thereafter. The susceptibility to *S. typhimurium* endotoxin was determined only at zero and 48 hours.

Discussion

Continued cortisone treatment may result in adrenal cortical decompensation. Abrupt withdrawal of cortisone also results in a period of relative adrenal suppression.[12,13] The experimental design used was thus aimed to produce a period of relative adrenal insufficiency subsequent to cortisone administration.

Assuming that such a status was produced, the results would appear to be in agreement with those cited in the literature review.

The *Esch. coli* endotoxin employed was prepared from a strain of organisms nonpathogenic for weaned mice[9]; a strain which may constitute part of the normal intestinal flora of man and animals.

We have demonstrated that the *Esch. coli* strain used, when living, has an enhanced capacity for invasion during and subsequent to cortisone administration.[9] Relative adrenal insufficiency may thus enable normally harmless microorganisms to produce sufficient amounts of toxin to cause toxic manifestations or death.

It remains to be demonstrated if similar results can be obtained using various stress factors instead of cortisone or by introducing the endotoxins into the intestinal tract.

Summary

The resistance of weaned mice to the effects of *Escherichia coli* and *Salmonella typhimurium* endotoxins was increased during administration of cortisone and markedly decreased subsequent to its abrupt withdrawal. The practical significance of these results in relation to disease production is discussed.

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A Macroscopic Tube-Agglutination Test for Leptospirosis

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THE ADVANCE of knowledge in the field of leptospirosis is closely correlated with methods of serological testing. Determination of the actual incidence of the disease in human and in animal populations is dependent upon accurate and simplified methods of detecting the presence of leptospiral antibodies in serums.

Three general types of serological tests have been developed. The complement-fixation technique developed by Gaehtgens,[1] Randall *et al.*,[2] and York [3] is a complex procedure which requires several carefully standardized reagents. Also, the complement-fixation (CF) test may not give adequate identification of the infecting leptospiral species.

The agglutination-lysis test of Schüffner and Mochtar,[4] using living antigen and characterized by both agglutination and lysis,* is a reliable test long used in the diagnosis of leptospirosis. Although highly sensitive, the agglutination-lysis test has certain disadvantages. There must be a constant supply of living leptospiral organisms and all readings of the test must be made by microscopic examination.

Stoenner [5] devised a macroscopic capillary tube-agglutination test for leptospirosis, using antigens prepared by suspending formalin-killed leptospiras in hypertonic, buffered sodium chloride solution. This capillary tube-agglutination test compared favorably in sensitivity and specificity with the standard microscopic agglutination-lysis technique.

Stoenner [6] and Hoag *et al.*[7] have described macroscopic plate-agglutination tests designed to permit easy and rapid classification of serums into positive and negative categories.

As yet, there are no leptospiral agglutinating antigens available from commercial or official laboratories for general use. It is the purpose of this paper to describe an agglutination test which is practical for use in laboratories not specializing in leptospiral research. This test has been used in the routine diagnosis of leptospirosis by Meyer,[8] DeLay *et al.*,[9] and others.

MATERIAL AND METHODS

Leptospiral strains which have been shown to be highly specific in antigenicity and which grow luxuriantly in culture mediums can be obtained from several laboratories. Species such as *Leptospira pomona*, *Leptospira canicola*, and *Leptospira icterohaemorrhagiae* are commonly encountered as the infecting organisms in leptospirosis of domestic animals in the United States.

The leptospiral strains used in this study were received from Dr. J. W. Wolff, Institute voor Tropische Hygiene, Amsterdam. They were the following: *L. pomona* (L. Australie C), *L. canicola* (Hond Utrecht IV), and *L. icterohaemorrhagiae* 20 Wijnberg (type AB).

Preparation of Antigen.—The basic culture medium consisted of the Schuffner modification of Verwoort's medium [10] to which was added 8 to 10 per cent of sterile rabbit serum. After inactivation, the medium was dispensed in 70-ml. amounts in 125-ml. Erlenmeyer flasks which were inoculated with 1 to 3 ml. of actively growing leptospiral culture and incubated at 32 C.

Cultures used for antigen production were selected at the height of growth, which was usually after five to seven days of incubation. Each flask was checked by dark field examination for density of growth, contamination, and any indications of spontaneous agglutination. Leptospiral conglomerations or "foci of multiplication" may be mistaken for agglutinated masses but, on closer observation, these foci of multiplication are seen to contain individuals which are actively motile and have detailed structure. Agglutinated masses of leptospiras gradually degenerate and their movements are sluggish or absent.

Cultures were killed and preserved by the addition of chemically pure formalin in sufficient quantity to make a final concentration of 0.5 per cent.

After being held 12 hours under refrigeration, the antigen was ready for use. If kept refrigerated in a dark place, this antigen was stable for at least three months.[11]

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* The lysis mentioned in this discussion denotes the absence of both agglutinated and uniformly distributed viable leptospiras. It is not the purpose of this paper to decide whether this result is due to dissolution of the organisms or due to their aggregation into the so-called "lysis balls."

According to Stoenner,[17] in the agglutination-lysis test, antigens with lower cell counts produced higher end point titers than antigens of high cell count. In the agglutination-lysis test the titer end point was determined by the microscopic examination of the supernatant fluid for the absence or lysis of living leptospiral organisms. Therefore, with high cell count antigens, more antibody was necessary to produce the same titer end point when compared with lower cell count antigens. With the agglutination-lysis test, standardization of antigen is of major importance.

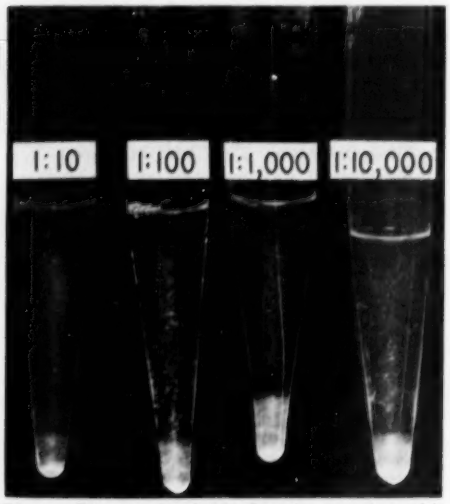

Fig. 1—The grossly visible floccule occurring with positive serum in the macroscopic tube-agglutination test.

In the performance of agglutination tests with killed antigens, the serum titer end point was determined by the presence of visible agglutination while residual antigen in the supernatant fluids was not considered. Antigens for use with the macroscopic agglutination test were standardized with the view of obtaining sufficient cell concentrations to form visible agglutination when antibodies are present. Formalized antigens having optical densities as low as half that of the standard antigen still produced grossly visible reactions at the same titer end point.

The Agglutination Test.—Tenfold serum dilutions in saline, from 1:10 through 1:10,000, in 0.5-ml. amounts, were placed in 3-ml. cone-point microchemical centrifuge tubes of 11-mm. diameter and 76-mm. length. To each tube of diluted serum, 0.5 ml. of antigen was added and the tubes were shaken. The serum-antigen dilutions were incubated for 12 hours at 37.5 C. and held six hours at room temperature, after which the reading was made.

A positive reaction was characterized by the appearance of grossly visible, fluffy clumps, and the degree of agglutination was graded from a 1+ to a 4+ reaction, depending upon the amount of floccule in the tube (fig. 1). A 1+ reaction was determined by dark field microscopic examination, while 2+ to 4+ reactions were macroscopically visible. In the negative tests, the 1:100 dilution was checked microscopically for evidence of slight agglutination or nonspecific lysis of the antigen. With positive serums, the end point titer was determined more accurately when dilutions not having visible reaction were checked microscopically. Controls for the test consisted of antigen added to the same dilutions of a known positive serum and to saline only. In interpreting the results of the macroscopic tube-agglutination test, a reaction occurring only in the 1:10 dilution was disregarded while a reaction in the 1:100 dilution was considered suspicious. Flocculation at dilutions of 1:1,000 and 1:10,000 indicates that the animal in question has experienced a leptospiral infection. To detect current infections, two or more serums must be tested for demonstration of an ascending titer. A practical approach to the diagnosis of leptospirosis would include serological results plus the clinical aspects of leptospirosis in that particular animal species.[12]

RESULTS

Evaluation of the macroscopic agglutination test was determined by five years of use in which 7,313 serum samples were tested (table 1). The high percentage of positive serums (bovine, 10 %; porcine, 18 %; canine, 20 %) was due to the fact that serums were presented for test by practicing veterinarians only when an undiagnosed disease problem was encountered. Very few of the samples were from routine serological surveys. Most of the herds with Leptospira-positive serums also presented the typical clinical signs of leptospirosis, such as hemoglobinuria and abortion in cattle, abortion in swine, and nephritis in dogs (table 2).

Additional evaluation of the agglutination test was secured by inoculation of

TABLE 1—Summary of Five Years of Testing with the Macroscopic Tube-Agglutination Test for Leptospirosis

Animal species	Tested (No.)	Positive (No.)	Positive (%)	Leptospiral species
Cattle	5,619	602	10.7	*L. pomona*
Swine	1,216	223	18.3	*L. pomona*
Dog	340	70	20.6	*L. canicola*
Man	62	1	1.6	*L. canicola*
Sheep	36	0	0.0	.....
Horse	21	4	19.0	*L. pomona*
Deer	10	0	0.0	.....
Goat	9	0	0.0	.....
Total	7,313	900	12.3	

TABLE 2—Correlation of the Positive Agglutination Reaction with Clinical Leptospirosis

Herd	Species	Phase of disease	Leptospiral species	Agglutination reaction					No. of isolations	Organism isolated
				Negative	1:10	1:100	1:1,000	1:10,000		
A	Swine	Chronic nephritis	*L. pomona*	19*	2	7	4	13	2	*L. pomona*
B	Swine	Chronic nephritis	*L. pomona*	11	6	12	15	45	14	*L. pomona*
C	Cattle	Acute hemoglobinuria	*L. pomona*	12	4	7	2	1	2	*L. pomona*
D	Cattle	Acute hemoglobinuria	*L. pomona*	5	2	2	4	4	1	*L. pomona*
E	Cattle	Acute hemoglobinuria	*L. pomona*	0	2	11	6	2	1	*L. pomona*
F**	Dog	Chronic nephritis	*L. canicola*	41	2	6	4	12	2	*L. canicola*

* Number of animals; ** all from one military base.

normal animals with known cultures of leptospiral organisms. The rate and extent of specific antibody formation was determined. As can be seen (table 3), all serums taken before inoculation failed to agglutinate the antigen, whereas after inoculation, all agglutinated with dilution end points that ranged from 1:100 in a calf that died of the acute disease to 1:20,000 in rabbits receiving multiple injections of antigen.

TABLE 3—Result of Agglutination Tests with Serums of Experimentally Inoculated Animals

Experimental animal	Preinoculation titer	Leptospiral species	Highest postinoculation titer	Days postinoculation
Pig 1	0*	*L. pomona*	1:10,000	22
Pig 2	0	*L. pomona*	1:10,000	24
Pig 3	0	*L. pomona*	1:10,000	27
Pig 4	0	*L. pomona*	1:10,000	22
Calf 1	0	*L. pomona*	1:100	10 (died)
Calf 2	0	*L. pomona*	1:10,000	15
Calf 3	0	*L. canicola*	1:10,000	14
Rabbit 1**	0	*L. pomona*	1:20,000	32
Rabbit 2	0	*L. canicola*	1:20,000	32

* Titer negative; ** 4 injections of antigen.

Serums were obtained from cattle known to have recovered from rhinotracheitis, and from other cattle that were convalescing from anaplasmosis. Serum samples known to agglutinate Brucella and vibrionic abortion antigens also were obtained and tested. Serums from swine vaccinated for hog cholera were tested. Serums were also obtained from dogs convalescing from distemper and hepatitis. Serums that agglutinated leptospiral antigens were included as controls. These results are summarized (table 4).

Serums containing antibodies to agents causing rhinotracheitis, anaplasmosis, brucellosis, vibrionic abortion, hog cholera, canine distemper, and canine hepatitis failed to agglutinate leptospiral antigens. It appears, therefore, that the macroscopic tube-agglutination test for leptospirosis is specific.

Cross reactions, similar to those described by Broom,[14] occurred with the macroscopic test. *Leptospira icterohaemorrhagiae* titers were observed with *L. pomona* infections but were much more pronounced in *L. canicola* infections. The cross reactions were usually of a lower magnitude when compared with the true infecting species, especially during the convalescent stages of the illness.

Comparison with the Agglutination-Lysis Test.—Selected cattle serums having various levels of *L. pomona* antibodies were tested by both the macroscopic tube-agglutination and the agglutination-lysis methods. In the agglutination-lysis test, a suspension of living leptospiras was mixed with tenfold dilutions of serum, incubated for four hours, and then examined by dark field examination.

There was a close correlation between

TABLE 4—Results of Leptospira Agglutination Tests with Serums of Animals Convalescing from Various Diseases

Disease	Animal species	Tested (No.)	Results with respective antigens		
			L. pomona	*L. canicola*	*L. icterohaemorrhagiae*
Rhinotracheitis	Cattle	17	0	0	0
Anaplasmosis	Cattle	21	0	0	0
Brucellosis	Cattle	8	0	0	0
Vibrionic abortion	Cattle	14	0	0	0
Hog cholera	Swine	25	0	0	0
Distemper	Dog	5	0	0	0
Hepatitis, infectious	Dog	4	0	0	0
Leptospirosis	Dog	24	0	1:10 to 1:10,000	0 to 1:1,000
Leptospirosis	Swine	42	1:10 to 1:10,000	0	0 to 1:100
Leptospirosis	Cattle	22	1:10 to 1:10,000	0	0 to 1:100

the results of the two tests, with serums having high or low end point dilution values matching closely. In about 15 per cent of the serums, the end point dilution value with the macroscopic agglutination method was one dilution lower than with the agglutination-lysis test. This discrepancy was alleviated when critical evaluation of the end point with the macroscopic method was determined with dark field examination in conjunction with the gross reading of the test.

SUMMARY

Details have been given for the preparation of antigens for use in the routine diagnosis of leptospirosis. *Leptospira pomona*, *Leptospira canicola*, and *Leptospira icterohaemorrhagiae* organisms were propagated in culture medium and formolized when maximum growth had been obtained. In performing the test, tenfold dilutions of the serum in saline were mixed with the appropriate antigens and incubated. A grossly visible floccule in the bottom of the cone-shaped tubes denoted a positive reaction.

An evaluation of the agglutination test was made by the routine testing of 7,313 serums during a five-year period. Most of the herds with Leptospira-positive serums also presented the typical clinical picture of leptospirosis. Experimental animals inoculated with Leptospira developed agglutination titers specific for the infecting organism.

The test was highly specific for leptospiral infections. Serums containing antibodies to various bovine, porcine, and canine diseases failed to agglutinate leptospiral antigens. The test compared favorably with the classic agglutination-lysis test.

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Rabies Street Virus in the Syrian Hamster

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SPECIFIC inclusion bodies present in the central nervous system of man and animals dying of rabies are called Negri bodies.[1,2] These are most frequently demonstrated within, but occasionally outside, the neurons. A definite diagnosis of rabies is made upon demonstration of these bodies in the neurons of the brain in stained smears or tissue sections.

These inclusions are eosinophilic staining bodies, usually spherical but also of other shapes, varying from 1 to 30 μ in size.[3] The inner structure shows basophilic-stained granules. The presence of Negri bodies is a criterion for diagnosis of rabies. Since the Syrian hamster is more susceptible than mice to rabies the following study was undertaken to see whether hamsters experimentally infected could have virus in their salivary glands during the incubation period and upon the appearance of rabid symptoms.

MATERIALS AND METHODS

The virus for the present study was obtained from a dog and a fox brain sent to this laboratory for routine examination for rabies. The hippocampus was removed from each brain aseptically, ground with alundum in a mortar, and diluted with physiological saline to a 20 per cent suspension. A 20 per cent suspension of the dog brain was injected (0.03 ml.) intracerebrally into each of six 3-week-old Syrian hamsters. These hamsters were kept in separate glass specimen jars, fed dog checkers and a bottle of water daily. A group of 6 hamsters was injected likewise but with the fox brain suspension and similarly isolated. The results are given in table 1. Hamsters infected with the dog brain virus suspension showed signs of furious rabies on the sixth day after exposure. The salivary glands from each hamster were removed aseptically, ground to a 20 per cent suspension in physiological saline, and each injected intracerebrally into 8 Swiss albino mice (0.03 ml. per mouse). The brain was removed from each hamster and the process was repeated. Hamsters exposed to the fox brain suspension also showed rabid symptoms on the sixth day. This technique was also used for the brain and salivary glands used for mouse inoculation. The results are given in table 2.

When inoculated mice showed symptoms of nervous system involvement, they were destroyed and each mouse brain was examined under an optical microscope and stained with Seller's stain.[4] The results are given in table 2.

TABLE 1—Response of 21-Day-Old Syrian Hamsters to a 20 per cent Suspension of a Rabid Dog and 20 per cent Suspension of a Rabid Fox Brain Inoculated Intracerebrally

Hamster (No.)	Amount injected (ml.)	Virus source	Symptoms and furious rabies (day)
1	0.03	Dog brain	6
2	0.03	Dog brain	6
3	0.03	Dog brain	6
4	0.03	Dog brain	6
5	0.03	Dog brain	6
6	0.03	Dog brain	6
1	0.03	Fox brain	6
2	0.03	Fox brain	6
3	0.03	Fox brain	6
4	0.03	Fox brain	6
5	0.03	Fox brain	6
6	0.03	Fox brain	6

TABLE 2—Response of Swiss Albino Mice Exposed Intracerebrally with Suspensions of Hamster Brain and Salivary Glands from Hamsters Exposed to the Dog Brain and Fox Brain Rabies Virus

	No. mice exposed	Symptoms in mice (days)	Negri bodies	Source of virus
Hamster brain	8	6–10	+	Dog brain
Hamster glands	8	6–10	+	Dog brain
Hamster brain	8	6–10	+	Dog brain
Hamster glands	8	6–10	+	Dog brain
Hamster brain	8	6–10	+	Dog brain
Hamster glands	8	6–10	+	Dog brain
Hamster brain	8	6–10	+	Dog brain
Hamster glands	8	6–10	+	Dog brain
Hamster brain	8	6–10	+	Dog brain
Hamster glands	8	6–10	+	Dog brain
Hamster brain	8	6–10	+	Dog brain
Hamster glands	8	6–10	+	Dog brain
Hamster brain	8	6–10	+	Fox brain
Hamster glands	8	6–10	+	Fox brain
Hamster brain	8	6–10	+	Fox brain
Hamster glands	8	6–10	+	Fox brain
Hamster brain	8	6–10	+	Fox brain
Hamster glands	8	6–10	+	Fox brain
Hamster brain	8	6–10	+	Fox brain
Hamster glands	8	6–10	+	Fox brain
Hamster brain	8	6–10	+	Fox brain
Hamster glands	8	6–10	+	Fox brain
Hamster brain	8	6–10	+	Fox brain
Hamster glands	8	6–10	+	Fox brain

From The Virus Laboratory, Live Stock Sanitary Service, University of Maryland, College Park.

SUMMARY

Syrian hamsters exposed to the dog brain virus showed furious rabies on the sixth day after exposure. Suspensions from the brain and salivary glands from these animals were injected intracerebrally into Swiss albino mice. These mice showed rabid symptoms between six and ten days and all brains upon microscopic examination contained numerous Negri bodies. Syrian hamsters exposed to the fox brain virus also showed furious rabies on the sixth day. The results were similar.

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The Susceptibility of Weaned Mice to *Escherichia Coli* During, and Subsequent to, Cortisone Treatment

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THE INFECTIOUS capacity of several representative species of bacteria and virus is greatly enhanced by cortisone treatment of the host.[17]

This includes microorganisms such as streptococci, pneumococci, micrococci, Brucella spp., typhoid bacilli, spirochetes, Corynebacterium spp., and poliomyelitis, influenza, and Coxsackie viruses. The list of hosts in which this enhancing effect has been demonstrated include mice, rats, guinea pigs, hamsters, rabbits, dogs, monkeys, men, and chicken embryos.[18]

Mogabgab and Thomas[9] have shown that cortisone treatment will render rabbits susceptible to group A hemolytic streptococci. Large intradermal inoculations of streptococci resulted only in vigorous local inflammation in the untreated control rabbit, while cortisone-treated rabbits developed no evidence of local reaction but succumbed later to a septicemia.

Experimental latent streptococcic infection in rabbits has also been reactivated by cortisone treatment to produce lethal septicemia.[8] According to Thomas,[18] similar results have been observed with other types of infection.

Adrenal insufficiency also causes an increase in susceptibility to bacterial infection, which is comparable to that resulting from cortisone treatment. Thus, Robinson *et al.*[13] have shown that adrenalectomized animals were rapidly overwhelmed when injected with pneumococci and that the resistance was restored by giving small doses of cortisone. This resistance to infections would again disappear when the dosage of cortisone was increased sufficiently.

Kass and Finland[8] and Robinson *et al.*[13] have studied the effects on infection of other adrenal corticoid substances. Seventeen-hydroxycorticosterone (compound F) appeared to have the same effect as cortisone, and corticosterone (compound B) caused a very slight enhancement of pneumococcic infection. Desoxycorticosterone combined with augmented salt intake did not appear to have any effect on the susceptibility of rabbits. Although cortisone has the capacity to interfere with resistance, it seems to have very little effect on acquired immunological resistance.[18]

The following experiments were designed to compare blood invasion by bacteria from the alimentary and respiratory tracts of normal untreated and cortisone-treated mice.

MATERIALS AND METHODS

Experimental Animals.—Weaned mice were obtained from a colony of Webster Swiss white mice. This particular mouse colony has an extended history of freedom from infection and from death losses. The mice were weaned when 3 weeks old, after which they were given Purina laboratory chow and fresh tap water, *ad libitum*.

Bacterial Suspensions.—*Escherichia coli* (0-101) was used in these experiments. Suspensions of this organism were prepared from 24-hour growth on brain-heart infusion agar (Difco). The organisms were washed off with physiological saline in such quantities as to give a suspension density of approximately 1×10^{10} microorganisms per milliliter. For exposure, approximately 0.01 to 0.02 ml. was placed either in the oral cavity or on the nasal openings during ether anesthesia.

Cortisone.—A saline suspension of cortone® acetate (cortisone acetate, Merck) was used in these experiments. The daily dosage was 2.5 mg. (0.1 ml. of a suspension containing 25 mg./ml.) intramuscularly in the hip region.

Experimental Procedure.—Two groups of mice, one of which received cortisone for three days and one for six days, were exposed to *Esch. coli* within one hour after the second injection of cortisone. A control group, which did not receive cortisone, was exposed to *Esch. coli* simultaneously with the two cortisone-treated groups.

Ten mice were selected by random from each group at various intervals subsequent to the first cortisone injection. These were then killed. Blood or lung tissue, or both, were cultured for the presence of bacteria and the lungs were examined for gross pathological alterations.

Bacterial Examination of Blood and Lungs.—The mice were killed with ether. The chest cavity of each dead or killed mouse was aseptically opened from the dorsal side. Approximately 0.25 ml. of blood was withdrawn from the arteria and adjacent large blood vessels with a sterile, fine, capillary pipette and transferred to a tube containing 10 ml. of brain-heart infusion broth.

Subsequently, the peripheral half of the dia-

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TABLE 1 — Hemocultures of Cortisone-Treated and of Control Mice Exposed Orally to Escherichia Coli

Type examination	Group of mice	Hours after first cortisone injection							
		0	24	48	60	72	96	120	144
Hemocultures	1, Control group, no cortisone	1/10*	0/10	1/10	0/10	0/10	0/10	1/10	1/10
	2, Cortisone, 0 through 144 hr.	0/10	0/10	1/10		3/10	2/10	2/10	
	3, Cortisone, 0 through 48 hr.	0/10	0/10		2/10	4/10	2/10	4/10	1/10

* Numerator, number of positive blood cultures; denominator, number examined.

phragmatic lobe of one lung was removed aseptically, minced in a sterile Petri dish, and transferred to a tube containing 10 ml. of brain-heart infusion broth.

The enriched culture tubes were inoculated at 37 C. for 24 hours, then streaked on blood plates and on eosin methylene blue plates which were examined after 24 and 48 hours of incubation at 37 C.

RESULTS

Most mice which received cortisone developed roughened coats and appeared unthrifty on the second or third day of cortisone injection; they remained in this condition throughout the experimental period.

Oral Exposure to Escherichia Coli.—The results of the hemocultures (table 1) indicate that cortisone treatment causes a higher incidence of bacteremia. The increase in incidence occurred during the fourth, fifth, and sixth day of cortisone injection and was even higher four days subsequent to withdrawal of cortisone.

Coliform bacteria were recovered from 21 of 27 positive blood cultures.

Intranasal Exposure to Escherichia Coli Under Ether Anesthesia.—Respiratory distress was not observed in any of the mice.

Results of the bacteriological and pathological examinations (table 2) suggest that mice which received cortisone developed a considerably higher incidence of bacteremia and less tendency to lung consolidation than control mice subsequent to pulmonary exposure to *Esch. coli*.

The incidence of bacteremia, which was similar in both groups receiving cortisone, remained approximately constant for several days subsequent to exposure. Lung consolidations observed in mice which received cortisone tended to develop later and were far less extensive than those seen in animals not receiving cortisone. However, the majority of lungs of cortisone-treated animals were hyperemic and edematous, and several showed small hemorrhages.

Bacteria were present in the lungs of a number of animals of all groups subsequent to *Esch. coli* exposure, but the incidence was lower in the control groups during the first two or three days after exposure. The great majority of positive blood and lung cultures proved to be pure cultures of *Esch. coli*. Cocci were recovered in a few cases.

DISCUSSION

The strain of *Esch. coli* used (0-101) in these experiments has previously been found to have no harmful effects on normal weaned mice when given orally in large doses.[11] Various strains of *Esch. coli* are normally established very early in life as part of the bacterial flora of the intestinal and upper respiratory tracts.[1] Oral exposure to *Esch. coli* in the present experiments was carried out in order to further insure the presence of *Esch. coli* at the time of, or subsequent to, cortisone injection.

TABLE 2—Bacteriological Examination of Blood and Lungs and Pathological Examination of Lungs of Cortisone-Treated and of Control Mice Exposed to Escherichia Coli by Pulmonary Route

Type examination	Group of mice	Hours after first cortisone injection						
		0	24	48	72	96	120	144
Hemocultures	1, Control group, no cortisone	1/10*	0/10	1/10	3/10	1/10	2/10	1/10
	2, Cortisone, 0 through 144 hr.	0/10	0/10	1/10	7/10	3/10	7/10	7/10
	3, Cortisone, 0 through 48 hr.	0/10	0/10	3/10	3/10	3/10	8/10	8/10
Lung cultures	1, Control group	0/10	1/10	3/10	5/10	2/10	8/10	8/10
	2, Cortisone, six days	2/10	0/10	2/10	9/10	6/10	8/10	9/10
	3, Cortisone, three days	2/10	0/10	5/10	9/10	5/10	7/10	5/10
Gross lung consolidation	1, Control group	0/10	0/10	0/10	3/10	7/10	7/10	7/10
	2, Cortisone, six days	0/10	0/10	0/10	0/10	2/10	3/10	4/10
	3, Cortisone, three days	0/10	0/10	0/10	0/10	2/10	3/10	3/10

* Numerator, number positive; denominator, number examined.

The increased incidence of "trespassing" of such microorganisms as *Esch coli* during, and subsequent to, cortisone treatment may well shed some light on the rather hazy concept of "potential pathogenicity" or "opportunism." These designations infer ability of disease production under certain conditions or whenever opportunities are offered.[19] Yet these "certain conditions" and various "opportunities," which might pertain to the microorganisms, to the hosts, or to both, have not been sufficiently defined.

Bacterial mutation of transformation from a nonpathogenic to a pathogenic form or stage is an accepted phenomenon but seems also to be related with conditional factors. In many instances, biological and serological differences can not be established between the normally inhabiting forms and their pathogenic forms.[2,20] Furthermore, it has often proved difficult or impossible to fulfill Koch's postulates with the "pathogenic forms."[10]

More or less specific host factors, such as infections, deficiencies, overexercise, and extremes of temperature and age seem to favor the invasion and establishment of pathogenic microorganisms as well as opportunists.

Most of these host factors have at least two characteristics in common: (1) stressing effect on the host and (2) a tendency to lower host resistance.[14] Various systemic infections in man, such as malignant diphtheria, cholera, and acute Streptococcus and Micrococcus septicemias are considered to produce severe stress,[7,14] and these and other infections have been claimed to produce a condition of relative corticoid deficiency in the host.[12]

According to Selye,[14] all stressors give rise to a triphasic general adaptation syndrome in the host. During the first stage (alarm reaction), there is increased production of corticoids, including cortisone, which is maintained at relatively high levels during the subsequent stage of resistance.[4] During the stage of exhaustion, the production of corticoids declines due to adrenal cortical failure.[14]

Both specific and nonspecific (crossed) resistance diminish during the "shock stage" and increase during the "counter-shock stage" of the alarm reaction. However, while specific resistance increases during the subsequent stage of resistance, the nonspecific resistance usually declines during this period:[5,6,14] *i.e.*, one stressing factor decreases the host tolerance to others. Both specific and nonspecific resistance diminishes during the stage of exhaustion.[14]

The experimental design used was aimed to produce: (1) relatively high, constant cortisone levels in group 2 corresponding to a stage of resistance; (2) a period of relative adrenal insufficiency in group 3 subsequent to cortisone administration.[15,16]

Assuming that these goals were attained, the results would appear to be in agreement with the observations described above and with those of Robinson *et al.*[13]

The importance of a delicately balanced cortisone level for resistance to infection has been demonstrated by Robinson's experiments. The process of invasion and infection may thus be considered an interaction between the host and the microorganism which can be "triggered" by pathogens under optimal host conditions and by opportunists when the adrenal function is unbalanced.

SUMMARY

Increased invasion of the blood stream by bacteria from the alimentary and respiratory tracts was observed in mice during, and subsequent to, cortisone treatment.

There was an inverse relationship between the incidence of blood stream invasion from the lung and the incidence of local inflammatory response in the lung.

The status of adrenal function as a determining factor for invasion by "potential pathogenic bacteria" is discussed.

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Comparative Titrations of Vesicular Stomatitis Virus in Various Animal Species and in Tissue Culture

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IN PREPARATION for work with foot-and-mouth disease virus, certain aspects of vesicular stomatitis virus (VSV) were investigated. These included a study of the comparative susceptibility to VSV of cattle, guinea pigs, Swiss albino mice, chickens, embryonating chicken eggs, and kidney tissue cultures. The literature contains limited observations and references on the comparative susceptibility of these hosts to VSV.[1-5, 12]

In comparisons of infective titers in various hosts, sources of virus and methods of inoculation are essential considerations.

MATERIALS AND METHODS

Preparation of the Virus.—The Indiana (I) type of bovine VSV (strain Fort. Lupton)[6] used in these studies had been propagated through six serial passages in the bovine species. The New Jersey (NJ) type of bovine VSV (strain Concan)[6,7] had been propagated through three serial passages in the bovine species until the time of this study, when it was passed twice in embryonating eggs and then returned to the bovine species for one passage. In the comparative titrations involving 7- to 10-day-old mice and embryonating chicken eggs, the virus used was grown in the chorioallantoic membrane (c.a.m.) of the embryonating chicken egg for two to five passages. In the other species and in tissue culture, bovine-passaged virus was used.

Each sample of infected, bovine tongue epithelium or c.a.m. was ground in a mortar with alundum, diluted to a 10 per cent suspension with tryptose phosphate broth and centrifugated at 2,000 r.p.m. for 20 minutes. To each milliliter of suspension was added 1,000 units of penicillin and 1 mg. of streptomycin. Subsequent tenfold dilutions were made in broth and used in the titrations.

Inoculation of Embryonating Chicken Eggs.—Groups of 5, 7- to 8-day-old embryonating chicken eggs were used for each dilution. Each egg received 0.2 ml. of inoculum into the allantoic cavity. Dilutions of virus used were 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9}. All injected embryos were incubated at 35 C. and observed for ten days. Embryo mortality within 24 hours after inoculation was considered to be due to nonspecific causes. The lethal dose$_{50}$ ($l.d._{50}$) was calculated by the method of Reed and Muench.[8]

Inoculation of Mice.—Groups of 5, 3- to 4-week-old Swiss albino mice were each inoculated with 0.03 ml. of the virus dilutions by the intracerebral route. Dilutions of virus used were 10^{-6}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9}. The mice were observed daily for 14 days for nervous symptoms, paralysis, and death. Death of mice within 24 hours after inoculation was considered due to trauma.

Additional groups of 5 suckling mice (7 to 10 days old) were inoculated with 0.03 and 0.05 ml. by the intracerebral and intraperitoneal routes, respectively. Dilutions of virus used were 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10}. The mice were observed daily for 14 days. The $l.d._{50}$ was calculated for both ages of mice by the method of Reed and Muench.[8]

Inoculation of Guinea Pigs.—Groups of 5 guinea pigs, weighing approximately 500 to 600 Gm. each, were inoculated by the intradermal tunnelling method in the metatarsal pads with 0.1 ml. of virus dilution per pad. Dilutions used were 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}. Observations of guinea pigs for VSV lesions were made at 48, 72, and 96 hours. The infective dose ($l.d._{50}$) was calculated[8] on the basis of ten test sites, two pads per guinea pig.

TABLE 1—Lethal Dose$_{50}$ and Infectious Dose$_{50}$ Titers of Indiana Type Bovine Vesicular Stomatitis Virus in Embryonating Chicken Eggs, Mice, Guinea Pigs, Tongues of Adult Cattle, and Tissue Cultures

Source of virus bovine tongue (anim. No.)	$l.d._{50}$* Eggs	$l.d._{50}$ Mice (3–4 wk.)	$l.d._{50}$* Guinea pigs	$l.d._{50}$ Tissue cultures	$l.d._{50}$ Adult bovine tongue
22	5.6	7.1	4.3	6.2	—**
27	7.3	6.8	4.3	5.9	—
28	5.7	7.3	4.3	5.9	—
30	7.4	7.5	4.5	5.9	—
31	5.8	7.3	4.3	5.7	5.7
32	7.8	8.1	5.5	6.5	7.5
33	7.3	7.4	5.0	7.2	6.7
34	6.7	7.3	4.5	6.5	7.7

* Expressed as log of reciprocal of dilution; ** not done.

From the U.S.D.A. Animal Disease and Parasite Research Branch, Plum Island Animal Disease Laboratory, Greenport, L. I., N. Y.

Captain T. H. Vardaman, U.S.A.F., was assigned to Plum Island while on extended active duty. His present address is the Regional Laboratory, U.S.D.A., ARS, Auburn, Ala.

[6] Isolated in 1949 in Concan, Texas.

TABLE 2—Lethal Dose$_{50}$ and Infectious Dose$_{50}$ Titers of New Jersey Type Bovine Vesicular Stomatitis Virus in Embryonating Chicken Eggs, Mice, Guinea Pigs, Tongues of Adult Cattle, and Tissue Cultures

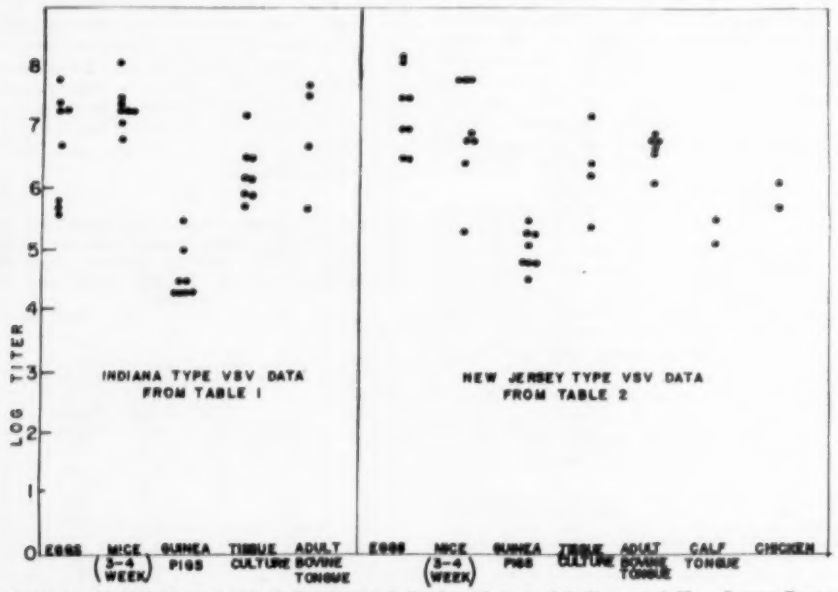
Source of virus bovine tongue (anim. No.)	l.d.$_{50}$* Eggs	l.d.$_{50}$ Mice (3–4 wk.)	i.d.$_{50}$* Guinea pigs	l.d.$_{50}$ Tissue cultures	i.d.$_{50}$ Chickens	i.d.$_{50}$ Calf tongues	i.d.$_{50}$ Adult bovine tongues
5	7.5	7.8	5.3	6.2	—**	—	6.8
6	8.2	6.9	5.1	5.4	—	—	6.8
9	8.1	7.8	5.5	6.4	5.7	5.1	6.7
10	7.5	7.8	4.8	7.2	6.1	5.5	6.9
12	6.5	6.8	4.8	—	—	—	6.6
15	6.5	6.4	4.8	—	—	—	—
16	7.6	5.3	4.5	—	—	—	—
20	7.0	6.8	5.3	—	—	—	6.1

* Expressed as log of reciprocal of dilution; ** not done.

Tissue Culture Inoculation.—Trypsin-dispersed epithelial cells of guinea pig kidney, grown for seven days in stationary tube cultures, were inoculated with 0.2 ml. of the virus dilution per 2 ml. of medium in each tube. Five tubes were used per virus dilution. The medium consisted of 50 per cent Hanks' salt solution, 30 per cent horse serum, and 20 per cent chicken embryo extract. Dilutions of virus used were 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} The cultures were incubated at 37 C. and observed for cytopathogenic effect at 24, 48, and 72 hours. The criterion of cytopathogenic effect of the virus was the appearance of plaquelike areas in the growing kidney epithelial cells in which the cells became rounded and the nuclei of such cells were pyknotic. The i.d.$_{50}$ was calculated on the number of tubes showing such an effect.[9]

Inoculation of Chickens.—In two instances, groups of 5, 8- to 10-month old crossbred Rhode Island Red roosters received 0.04 ml. of the virus dilution subcutaneously in the tongue. Dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} were used. The presence or absence of vesiculation was noted and final readings made at 48 hours.[10]

Inoculation of Bovine Tongue.—Four dilutions of virus were employed and five sites were inoculated with each dilution in each of two tongues from adult cattle by the intradermalingual route according to Henderson's method.[11] Steers between 18 and 24 months old were used. Exceptions were two groups of 5 mixed grade, dairy



Graph 1—Distribution of Lethal Dose$_{50}$ and Infectious Dose$_{50}$ of Indiana and New Jersey Types of Vesicular Stomatitis Virus in Various Hosts.

TABLE 3—Titration of Chorioallantoic Membrane Infected with New Jersey Type Vesicular Stomatitis Virus in 8- to 10-Day-Old Mice and Embryonating Chicken Eggs

Sample No.	Number of egg passages of inoculum	Route of inoc. for mice	Dose vol. in ml. for mice	Age of mice (days)	l.d.$_{50}$† in mice	l.d.$_{50}$ in eggs
1	5	IC*	0.03	8	9.3	8.3
1	5	IP**	0.05	8	8.3	8.3
2	4	IP	0.05	8	9.3	9.0
3	4	IC	0.03	9	8.6	9.0
3	4	IP	0.05	9	8.0	9.0
4	5	IC	0.03	10	7.9	8.3
4	5	IP	0.05	10	6.8	8.3

* IC intracerebral; ** IP intraperitoneal; † expressed as log of reciprocal of dilution.

calves between 4 and 6 months old, which were inoculated by the intradermalingual route at two sites per dilution, making a total of ten sites per calf. An inoculum of 0.1 ml. of virus dilution was used per site. Dilutions of virus inoculated into cattle and calves were 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}. The tongues were examined for vesiculation at 24 and 48 hours.[11]

RESULTS

The results of the comparative titrations of VSV I type bovine tongue epithelium in various mediums are given (table 1) and for the NJ type bovine VSV (table 2). Tables 3 and 4 show the l.d.$_{50}$ values obtained in limited comparative titrations of c.a.m. infected with both types of VSV, in 7- to 10-day-old mice and in embryonating chicken eggs. The distribution of the l.d.$_{50}$ and i.d.$_{50}$ values of VSV of bovine origin in the various mediums are given (graph 1).

All values in tables and in figures are converted to a 0.2-ml. quantity of virus inoculum for uniformity in comparison. Tongues from adult cattle and tissue cultures gave approximately the same i.d.$_{50}$ values. The calf tongue i.d.$_{50}$ values in the two tests reported here were lower than the titer with the tongues from adult cattle but paralleled the guinea pig pad and chicken tongue titers. Nagel[12] has made reference to the propagation of VSV in 4- to 10-day-old mice by the intraperitoneal route. A limited study was made on the comparative titration of NJ and I types of VSV in 7- to 10-day-old mice inoculated by the intraperitoneal and the intracerebral routes and in embryonating chicken eggs. A trend was indicated toward the greater sensitivity of the younger mouse. The young mice responded to VSV infection by developing encephalitis, quadriplegia, or hemiplegia of the hindlegs followed by death. Limited comparison between 3- to 4-week-old mice with older mice up to 6 months showed little difference in susceptibility when inoculated intracerebrally.

TABLE 4—Titration of Chorioallantoic Membrane Infected with Indiana Type Vesicular Stomatitis Virus in 7- to 9-Day-Old Mice and Embryonating Chicken Eggs

Sample No.	Number of egg passages of inoculum	Route of inoc. for mice	Dose vol. for mice (ml.)	Age of mice (days)	l.d.$_{50}$† in mice	l.d.$_{50}$ in eggs
1	2	IP*	0.05	7	10.0	7.5
2	2	IC**	0.03	9	9.1	7.5
2	2	IP	0.05	9	8.4	7.5
3	3	IC	0.03	8	9.3	8.4
3	3	IP	0.05	8	8.3	8.4
4	2	IC	0.03	8	9.3	7.5
4	2	IP	0.05	8	9.1	7.5

* IP intraperitoneal; ** IC intracerebral; † expressed as log of reciprocal of dilution.

SUMMARY

1) New Jersey and Indiana types of bovine vesicular stomatitis virus (VSV) were titrated in tongues from calves and adult cattle, and in embryonating chicken eggs, Swiss albino mice, guinea pigs, chicken tongues, and in tissue cultures of guinea pig kidney cells.

2) The 3- to 4-week-old mice, inoculated intracerebrally, and the embryonating chicken eggs were found to be the most sensitive and gave high l.d.$_{50}$ values. Tongues from adult cattle and tissue culture i.d.$_{50}$ were of about equal value and came next in order of sensitivity. Calf and chicken tongue titrations gave lower i.d.$_{50}$ values than the preceding mediums. The guinea pig gave the lowest values obtained in the test series.

3) Titrations of suspensions of chorioallantoic membranes infected with the New Jersey and Indiana types of VSV were made in 7- to 10-day-old mice inoculated intracerebrally and intraperitoneally, and in embryonating chicken eggs. These studies were not extensive, but yielded l.d.$_{50}$ values greater in the mouse than in the egg when titrating Indiana type VSV. This difference was not obtained when titrating New Jersey type VSV. A trend was observed toward greater sensitivity to VSV in the suckling mice.

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Colonial and Antigenic Variations of *Vibrio Fetus*

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THE ANTIGENIC relationships of strains of *Vibrio fetus* isolated from various host species have not been clearly defined. Reported serological studies of this organism have devoted little attention to the possible existence of colonial variation with its usual concomitant antigenic changes, phenomena known to be common in many bacterial species.

By use of the electron microscope, Rhoades [10] observed morphological variation of *V. fetus* on succeeding transfers. This consisted of filamentous organisms and appearance of granular changes in the cytoplasm. The organisms predominating in young cultures were described by Binns and Fincher [1] as short, comma-shaped, and S-shaped forms, and those of the older cultures as long spirals. Plastridge *et al.*[7] described *V. fetus* colonies on thiol agar as being convex, 1 to 3 mm. in diameter, and varying from translucent to opaque in appearance.

Cultures maintained on semisolid agar tended to become agglutinable with normal bovine serum and failed to agglutinate proportionately to a higher titer with known positive serum. Morse and Ristic [6] showed that *V. fetus* antigens prepared from strains following a two- to 12-day residence in the guinea pig proved to be slightly superior in agglutinability with specific antiserums as compared to those prepared from the original or nonpassaged strains. On the basis of cross-agglutination studies, a considerable degree of antigenic heterogeneity has been observed among *V. fetus* strains.[2, 9, 12]

These observations suggested the possibility of colonial variation in this organism, thus indicating the need for an investigation of this phenomenon as a prerequisite to any further study of its antigenic structure. Upon examination of stock cultures, maintained in semisolid medium, Herzberg and Ristic [5] observed a number of such variants when cultures were plated on solid medium and observed by indirect, oblique light. The present report extends those observations to the bacteriological, biochemical, and serological characteristics of the variants isolated from stock cultures of selected strains of bovine, ovine, and human origin.

MATERIALS AND METHODS

The histories of the ten strains studied are shown (table 1). All strains were representative of *V. fetus* on the basis of their cultural, morphological, and biochemical characteristics. The organisms had been maintained in the laboratory for periods of 1 to 3 years by transfer at monthly intervals in Albimi * broth, containing 0.16 per cent agar.

To study colony morphology, cultures were streaked on solid Albimi mediums (containing 2% agar) so as to obtain discrete colonies. After incubation for 72 hours at 37 C. in an atmosphere of 10 per cent CO_2, the colonies were examined under a 10 to 20 magnification using oblique, transmitted light, according to the method of Henry.[4] The uptake of 1:1000 crystal violet by colonies was also employed to differentiate variants as described by White and Wilson.[13]

The fermentative characteristics of the variants were studied in cystine trypticase semisolid agar ** containing 1 per cent of the carbohydrate with phenol red as the indicator of acid production.

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* Albimi Laboratories, Brooklyn, N. Y.

** Baltimore Biological Laboratories, Baltimore, Md.

TABLE 1—Sources of Vibrio Fetus Cultures

Strain	Origin	Rate of isolation	Recovered by
U.F.	Bovine aborted fetus, Florida	1954	M. Ristic
F.	Bovine cervical mucus, Florida	1955	F. H. White
426	Bovine aborted fetus, Florida	1954	M. Ristic
D12	Bovine aborted fetus, Florida	1953	M. Ristic
3630	Bovine aborted fetus, Maryland	1955	A. H. Frank
543	Ovine aborted fetus, Maryland	1952	A. B. Crawford
K	Ovine aborted fetus, Wisconsin	1953	T. Kowalczyk and J. Simon
53D	Ovine aborted fetus, Colorado	1952	W. R. Graham
1134	Human blood, Massachusetts	1953	Peter Bent Brigham Hospital *
1154	Human blood, Massachusetts	1953	Boston City Hospital *

* Received through Miss E. O. King, C.D.C., U.S. Public Health Service.

Fig. 1—Cells of smooth colonial type of Vibrio fetus (U.F.) in gram preparation, appearing as short, curved, comma-shaped and incomplete S-shaped forms. x 900.

Catalase activity was determined on washed suspensions of Vibrio obtained after 60 hours' growth on solid Albimi agar. The density of the suspension was adjusted to a reading of 32 per cent light transmission in a Leitz photoelectric colorimeter approximating the No. 2 McFarland standard. Ten milliliters of suspension was placed in a 16 x 150 mm. culture tube equipped with a rubber stopper from which a gas delivery tube extended for 20 cm. into an inverted culture tube, of similar size, filled with water and situated in a water bath for convenience. Ten milliliters of 3 per cent H_2O_2 was added to the tube containing the bacterial suspension and the system was closed. After 30 minutes at room temperature, the amount of oxygen produced by catalase activity was recorded as the number of milliliters of water displaced in the inverted tube. Since temperature was not controlled precisely, all comparisons between cultures were made simultaneously under the same experimental conditions.

Antiserums were prepared by inoculating rabbits intravenously four times during a 21-day period with dense, living, suspensions obtained from 72-hour Albimi agar cultures. Agglutinating antigens consisted of washed organisms obtained from growth on solid medium suspended in saline

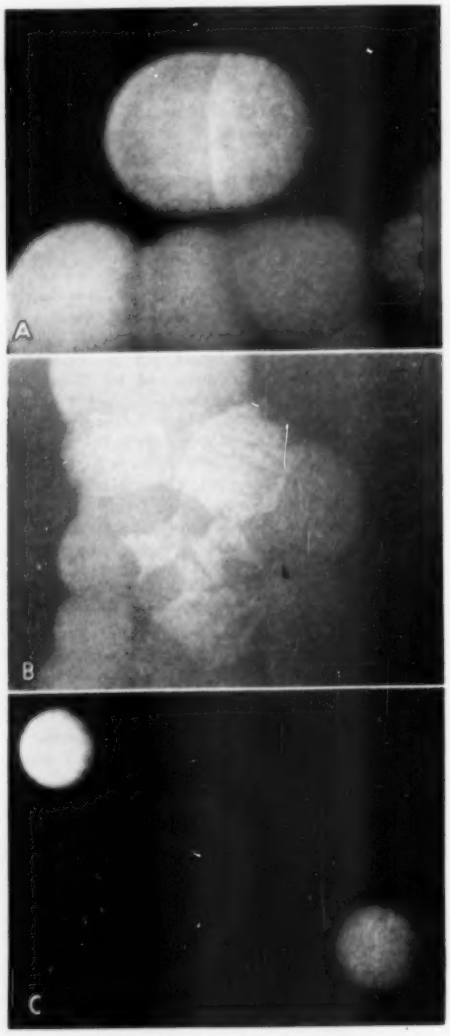

Fig. 2—Strains of Vibrio fetus.

A—Sectoring in the confluent growth of smooth and very rough colonies of Vibrio fetus, bovine strain U.F. Note opaque, granulated VR sector as opposed to the absence of granulation in the S colonies.

B—Ovine strain 53D, showing the contrast between VR (lighter colored colonies) and S colonies when they are immediately adjacent.

C—Single VR (lighter color) and S colony of human strain 1134. x 20.

containing 0.3 per cent formaldehyde. The antigens were stored as dense suspensions and were diluted for the agglutination tests with formaldehyde-saline to a density of 45 per cent light transmission in the Leitz photoelectric colorimeter. The tube agglutination test employed was described previously.[11] The stability of the suspension in the presence of neutral aqueous acriflavin * was studied as follows: Equal amounts of bacterial suspension and dilutions of the dye were placed in tubes and incubated at 37 C. for 48 hours after which the suspensions were examined for agglutination. The final concentrations of the dye which were employed ranged from 1:100 to 1:6400. Agglutinability of the antigens in normal bovine serum was studied by use of serum from a 14-month virgin heifer which came from a dairy herd having a satisfactory breeding record.

RESULTS

Colonial and Cellular Variations.—All variants described below were found in *V. fetus* cultures U.F. and 53D, which were studied intensively. Other strains studied revealed the presence of two or more of the same variant types.

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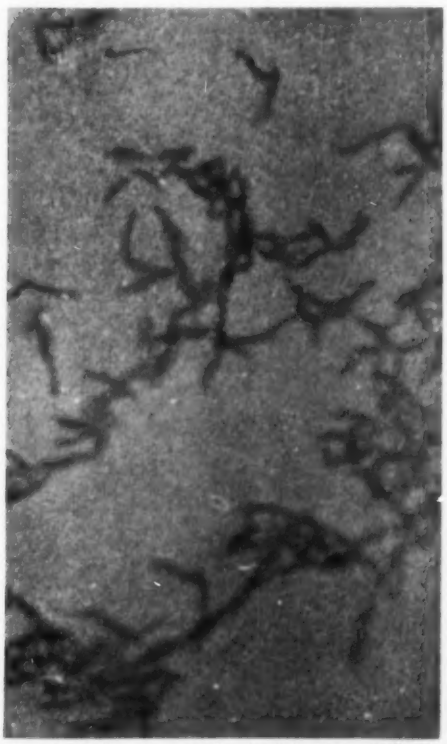

Fig. 3—Cells of mucoid colonial type of Vibrio fetus (53D) in gram preparation, appearing thicker and twice the length of the organisms of S type. x 900.

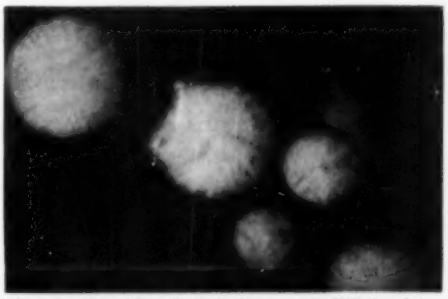

Fig. 4—Mucoid colonial forms of Vibrio fetus (U.F.) with yellowish brown central portion and light blue periphery, having radiating appearance of the surface as observed under procedure described. This is the largest colony type observed. x 20.

a) Smooth (S).—These were bluish, raised, entire edge, translucent colonies with very little internal granulation. The individual cells appeared as thin, curved, comma-shaped, and incomplete S-shaped forms, 1.5 to 2.0 μ in length (fig. 1, 2).

b) Rough (R).—These were similar to the S type except for whitish opaqueness due to marked granulation within the colonies. The cells appeared thicker and almost twice the length of the organisms of the smooth type.

c) Cut Glass (CGl).—These were brownish-green colonies, occasionally irregular edge, with the appearance of a cut-glass surface with large facets. The organisms of this type were distinctive long, spiral forms, which occasionally extended halfway across the microscopic field of the oil immersion objective (fig. 5, 6).

A common characteristic of these three variant colonies was their watery consistency, demonstrable by a tendency of the colony to flow away from the needle when touched.

d) Mucoid (M).—These were larger than previous colonial forms, with a yellowish brown central portion with light brown or blue periphery and, occasionally, a radiating appearance on the surface. The colonies adhered to the needle when picked. The cells appeared similar to the R forms (fig. 3, 4).

e) Very Rough (VR).—These were grayish white, flat, and opaque, occasionally

TABLE 2—Characteristics of Vibrio Fetus Variants

Characteristic studied	Strains							
	53D (Ovine)				U.F. (Bovine)			
	Variants							
	S	CGl	M	VR	S	CGl	M	VR
Aggl. bovine normal serum [1]	0	40	80	160	0	40	40	160
Aggl. neut. acriflavin [1]	200	800	800	6400	200	800	1600	3200
Aerobic growth [2]	−	+	+	+	−	+	+	+
Catalase activity [3]	37.1	32.0	30.0	0.7	38.8	30.2	28.0	0.4
Motility [4]	Active	Weak	Weak	Very weak	Active	Weak	Weak	Very weak
Crystal violet uptake [5]	None	Red violet	Red violet	Violet	None	Red violet	Red violet	Violet

[1] Expressed as the reciprocal of the final dilution showing complete agglutination; [2] growth on solid medium without replacement of 10 per cent of the air with CO_2; [3] milliliters of water displaced by oxygen produced under the described procedure (*see* Methods); [4] observed in hanging drop preparations; [5] after 15 seconds' exposure to 1:1000 aqueous crystal violet.

with an irregular surface (fig. 2). These colonies maintained their integrity when touched, and upon pressure with a needle gave an appearance of a waxy consistency with fragmentation into large pieces. Such colonies were difficult to emulsify into homogenous suspensions in saline. The VR variant, as a rule, was most stable on solid medium; however, reversion to M, CGl, and S colonies occasionally occurred after repeated subculture in broth.

The organisms of the VR type differed from the S type in being slightly longer, thicker, straighter, and having internal granulation.

A comparison of the colonial variants in regard to the various characteristics studied is shown (table 2). The three non-

Fig. 5—The cells of cut-glass colonial type of Vibrio fetus (53D) in gram preparation, appearing as distinctive, long spiral forms. x 900.

smooth forms were agglutinated readily in 1:40, 1:80, and 1:160 dilutions of normal bovine serum, while the S types were not agglutinated in dilutions of 1:10, the lowest dilution tested. The S forms were inagglutinable in acriflavin dilutions above 1:200 while CGl and M types agglutinated in dilutions of 1:800 and 1:1600, and type VR exhibited this reaction in dilutions of 1:3200 and 1:6400. The amounts of O_2 liberated from peroxide by VR forms in the catalase activity test did not exceed 1.00 ml., while the other three forms varied between 28.0 and 38.8 ml. of water displaced in 30 minutes. On moist, solid, and semisolid Albimi mediums, the three nonsmooth variants grew in a normal atmosphere, while S forms required replacement of 10 per cent of the air with CO_2 in order to develop. Although all the variants were motile in saline preparations of 72 hours of growth, these differed in degree of activity. The colonies of VR type appeared violet after exposure to 1:1000 crystal violet as exposed to lack of dye uptake by the smooth forms after exposure to the dye. The other variants displayed color variations of blue-red shades after similar treatment.

The fermentative abilities of the variants were tested with the following carbohydrates: maltose, sucrose, mannitol, galactose, lactose, xylose, sorbitol, dextrose, and rhamnose. In no instance was there an indication that production of acid had occurred in any of the cultures.

Results of cross-agglutination reactions among variants of *V. fetus*, strains 53D, (ovine origin) and U.F. (bovine origin), are shown (table 3). Smooth antigens agglutinated with antiserums of the S, M, and CGl variants of their own strain but failed

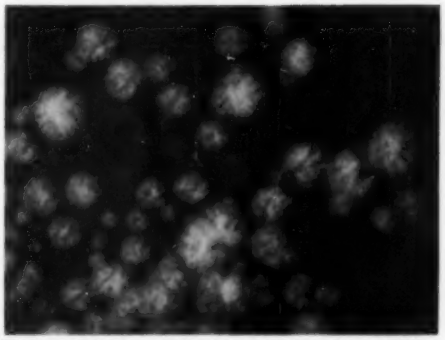

Fig. 6—Cut-glass colonies of Vibrio fetus (U.F.) appear brownish green having surface like cut glass with large facets when observed under the conditions described. x 20.

to cross react with any antiserums prepared against the variants of the heterologous strain. On the other hand, S antigens failed to agglutinate with VR antiserums of the homologous strains. A considerable degree of cross reaction was exhibited when nonsmooth variants were exposed to antiserums prepared against any variant of either homologous or heterologous strain. The M variants of both strains cross reacted to almost the identical level.

Discussion

The examination of stock strains of *V. fetus* showed that most cultures contained a high percentage of nonsmooth variant forms. These cultures had been maintained in semisolid Albimi broth and transferred monthly, using large inoculums. Such a procedure would have favored the selection of mutant types if any sort of selective differential existed between variants. The nonsmooth variants which were

TABLE 3—Cross Agglutination Among Variants of Two Vibrio Fetus Strains

Antigen		Antiserum							
		53D (Ovine origin)				U. F. (Bovine origin)			
Strain	Variant	S	VR	M	CGl	S	VR	M	CGl
53D	S	* 2560	0	640	320	0	0	0	0
	VR	40	20480	20480	320	40	160	2560	160
	M	80	20480	20480	640	160	160	10240	P1280
	CGl	640	1280	160	2560	160	320	5120	P2560
U.F.	S	0	0	0	0	2560	0	640	640
	VR	20	20480	20480	640	80	5120	160	40
	M	640	10240	10240	1280	80	640	10240	1280
	CGl	P1280	640	160	P2560	160	P640	1280	5120

* Number indicates the reciprocal of the highest dilution showing complete agglutination. Homologous titers are underlined.

tested grew equally well under either normal aerobic conditions or in an environment in which the atmosphere was diluted with CO_2. The smooth organisms failed to grow under normal aerobic conditions. This factor would have been sufficient to favor the selection of the nonsmooth types, which through repeated transfers and maintenance at room temperature would have gained ascendancy.

Considering the long periods of *in vitro* maintenance of these cultures, it would be reasonable to assume that the variants here described may have represented secondary mutants from nonsmooth forms rather than first-step variants from the normal, smooth form. Thus, future studies on freshly isolated strains are planned to determine the state of the organism *in vivo*, the direction of dissociation, and the nature and antigenic configuration of the intermediate types. This would then lead to the development of more effective methods of serological diagnosis and strain typing.

No statement can be made as to the mechanism of the effect of the added CO_2 as necessary for the growth of smooth variants. Whether this effect is due to a decreased O_2 tension or to the increased CO_2 tension, or a combination of both, remains to be tested.

It is apparent from data reported herein that confusing results in the field of diagnostic serology, and the studies of antigenic cross relationships among strains, may have resulted from the use of cultures in various stages of dissociation for production of specific serums and test antigens. In view of the differences in antigenic structure, which can occur in the mutation of S to R, as demonstrated in Salmonella, Brucella, and in the *V. fetus* strains described above, the serological heterogeneity and cross relationships in *V. fetus* cultures described in the literature should be scrutinized carefully. The antigenic relationships between *V. fetus* strains of different sources and animal species can best be clarified by a strict control of the various mutant types in the preparation of antigens and antiserums.

The S variants studied were the most strain specific in their agglutinability. The nonsmooth variants, in particular the M forms, demonstrated a considerable degree of cross reaction with the heterologous strain. In fact, the antigenic relationships between the S and the three nonsmooth forms arising from the same strain, as judged by the agglutination test, were less marked than the relationship shown between the M form of a strain of bovine origin and the corresponding variant type of another strain of ovine origin. This observation suggests that S→M→R dissociation results in loss of type specificity and a gain of an antigenic homogeneity in which the antigenic structures of *V. fetus* strains of different host origin come into apparent relation. Similar study on greater numbers of *V. fetus* strains should be conducted to ascertain the possibility of a common antigenic pattern of their M forms.

The agglutinability of variants in normal bovine serum is of considerable significance from the practical viewpoint. The occurrence of spontaneously agglutinating forms (such as the VR type) in cultures extensively used for antigen in agglutination tests may have resulted in misinterpretations and uncertainty of test results. Cell morphology under these circumstances is not a sufficient criterion in determining the predominant variant type. The easily recognizable long filament forms generally considered as undesirable for antigenic preparations behaved serologically closer to S forms than did the relatively short forms of the VR type. The acriflavin agglutinability appears, at present, as a most practical means, in addition to colonial differentiation, in examining the dissociative stage of a *V. fetus* culture.

There were apparently no differences in fermentative ability among the variants, since they were all inactive on the carbohydrates tested. The negligible catalase activity demonstrated by the VR form of the two strains is of interest in regard to the possible relationship of VR variants arising from "true" *V. fetus* strains to non-catalase producing "nonabortive" vibrios.[2]

SUMMARY

Examination of stock cultures of *Vibrio fetus* of bovine, ovine, and human origin, using oblique, indirect transmitted light, revealed the presence of one smooth and four distinctive nonsmooth colonial types with correlated cell morphology. The nonsmooth types were agglutinated with normal bovine serum in dilutions of 1:40 and

1:160 and with neutral acriflavin in dilutions of 1:800 to 1:6400. The smooth type required an atmosphere diluted with CO_2 for growth, while the other types grew equally well under normal atmospheric conditions. The uptake of 1:1000 crystal violet, low motility, and negligible catalase activity was demonstrated by very rough types.

On the basis of cross-agglutination reactions, strain specificity was shown by smooth types, while cross reaction was manifested among rough variants of homologous and heterologous strains.

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Notes on the Encapsulation of *Dirofilaria Immitis* in the Mosquito *Aëdes Aegypti*

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ALTHOUGH the encapsulation of filaria parasites in the mosquito host is a rather commonly observed phenomenon, very little quantitative estimation of the intensity of encapsulation has been reported. Kartman [2] presented some details of the encapsulation of the dog heartworm, *Dirofilaria immitis*, in *Aëdes aegypti*, *Culex quinquefasciatus*, and *Anopheles quadrimaculatus* and concluded that the phenomenon was of small quantitative importance to the extrinsic cycle of the parasite under experimental conditions. Other factors were shown to be much more important as limiting conditions for the intraculicid development of the filaria larva. Although this finding lent support to the view of Brug,[1] who suggested that filarial encapsulation is not a successful means of defense by the host, it was also noted [3] that encapsulation occurred more consistently in some mosquito hosts (e.g. *A. aegypti*) than in others.

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A review of some of the pertinent literature on filarial encapsulation is presented by Kartman.[4] The present communication offers quantitative data on encapsulation in *A. aegypti* in more detail than has hitherto been published.[5]

The materials, methods, and other procedures pertaining to these experiments already have been presented in detail.[5] It is sufficient to state that, after feeding upon an infected dog, the female mosquitoes were confined to small cardboard cages placed in an enamel pan lined with moist cotton. They were allowed to feed on sliced apple thereafter, and were kept in an air-conditioned insectary with the temperature held at approximately 27 C. and relative humidity of about 80 to 90 per cent. A number of individuals were dissected each day up to 20 days after the infectious blood meal, and their filaria infections were analyzed.

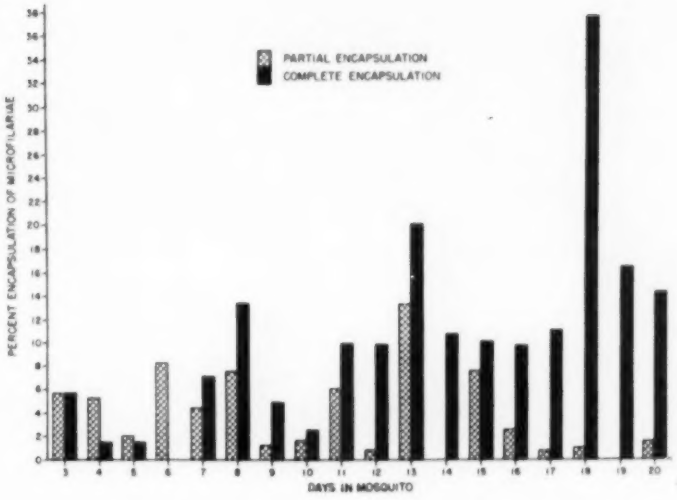
DISCUSSION AND RESULTS

A summary of the major portion of the data on encapsulation is presented in table 1. No encapsulation was observed until the third day after the infectious meal. Throughout the 20 days, only microfilariae or microfilaria-like forms and "sausage stage" larvae were found encapsulated. The bulk of encapsulated parasites were seen in the malpighian tubules, usually

TABLE 1—Encapsulation of Dirofilaria Immitis in Aëdes Aegypti

Days in mosquito	Number ♀♀ dissected	♀♀ with encap. forms		No. filariae in: All ♀♀	No. filariae in: ♀♀ with encap. forms	Microfilariae encapsulated in:* Mlpgh. tubules		Midgut		Haemocoele		Encapsulated "Sausage stage"*	
		(No.)	(%)			(No.)	(%)	(No.)	(%)	(No.)	(%)	(No.)	(%)
1	9	0	0.0	193	.....	....	.....	....	....	....	....	....	....
2	10	0	0.0	285	.....	....	.....	....	....	....	....	....	....
3	7	1	14.2	230	51	6	11.7	0	0.0	0	0.0	0	0.0
4	13	5	38.4	529	239	17	7.1	0	0.0	0	0.0	0	0.0
5	14	5	35.7	322	186	7	3.7	1	0.5	0	0.0	0	0.0
6	8	3	37.5	189	48	4	8.3	0	0.0	0	0.0	0	0.0
7	13	3	23.0	284	111	13	11.7	0	0.0	0	0.0	0	0.0
8	11	3	27.2	311	52	11	21.1	0	0.0	0	0.0	1	1.9
9	14	7	50.0	367	216	14	6.4	0	0.0	0	0.0	1	0.4
10	7	2	28.5	258	112	5	4.4	1	0.8	0	0.0	0	0.0
11	13	8	61.5	361	259	42	16.2	0	0.0	0	0.0	4	1.5
12	11	5	45.4	180	111	12	10.8	0	0.0	0	0.0	0	0.0
13	11	3	27.2	160	15	5	33.3	0	0.0	0	0.0	0	0.0
14	11	4	36.3	149	46	5	10.8	0	0.0	4	8.7	0	0.0
15	14	5	35.7	131	39	7	17.9	0	0.0	1	2.5	0	0.0
16	15	8	53.3	159	112	14	12.5	0	0.0	3	2.6	1	0.8
17	16	8	50.0	197	126	15	11.9	0	0.0	0	0.0	0	0.0
18	15	8	53.3	149	96	37	38.5	0	0.0	1	1.0	0	0.0
19	9	4	44.4	148	73	12	16.4	0	0.0	3	4.1	0	0.0
20	8	5	62.5	99	63	10	15.8	0	0.0	1	1.5	0	0.0
Totals	229	87	37.9	4701	1,955	236	12.0	2	0.1	13	0.6	7	0.3

* Based on filariae in mosquitoes showing encapsulated forms.



Graph 1—The type of encapsulation of Dirofilaria immitis microfilariae shown as percentages during a period of 20 days in Aëdes aegypti (no encapsulation occurred until the third day).

lying in the distal portions and quite often in saclike expansions of the tubules. Some encapsulated forms were found in the midgut and appeared no different from the type of encapsulation seen in the tubules. Encapsulated microfilariae were also found in the haemocoele (table 1) but only during the latter portion of the observation period. These were always found among the tracheoles surrounding the outer wall of the midgut.

The percentage of mosquitoes with encapsulated filariae increased with time. Similarly, the percentage of encapsulated filariae increased toward the termination of the time period selected. It should be pointed out that the majority of *D. immitis* microfilariae in *A. aegypti* were found to remain virtually unchanged during the 20-day period under the present experimental conditions. Very few of them initiated development to later larval stages. Whether or not this factor favored encapsulation is not known, but the hypothesis should be considered since in other mosquito hosts, in which most of the filariae developed to infective larvae, little or no encapsulation was seen under the same conditions.[3] It may be possible that microfilariae are more susceptible to encapsulation in an unfavorable mosquito host than are the later larval stages, and that this reaction increases in intensity the longer the microfilariae survive and remain unchanged. On the other hand, it is known that adverse temperature conditions favor encapsulation of later larval stages in mosquitoes.[2]

The encapsulation of the microfilariae was either partial or complete. Partially encapsulated parasites were affected at various places on their bodies, but the free portions of the worm showed movement. Completely encapsulated filariae evinced no movement whatsoever. Data on the percentage of partially and completely encapsulated microfilariae during the 20-day period (graph 1) suggest that the percentage of complete encapsulation increased directly with the length of time spent by the parasites in the mosquito. It would be of interest to observe the extent of encapsulation in mosquitoes over a longer period than is shown here. As far as can be determined, no knowledge exists regarding the ultimate fate of an encapsulated filaria parasite in the mosquito host.

Summary

Data are presented on the encapsulation of *Dirofilaria immitis* in *Aëdes aegypti*. Encapsulation occurred mainly on microfilariae and rarely on "sausage stage" larvae. The intensity of encapsulated parasites, and the number of mosquitoes with encapsulated forms, increased with the passage of time. Similarly, the percentage of completely encapsulated parasites increased as their stay within the mosquito lengthened. The data suggest that, in an unfavorable mosquito host and under the

conditions of the experiment, encapsulation of the unchanged microfilaria was favored in direct relation to the length of time spent in the host.

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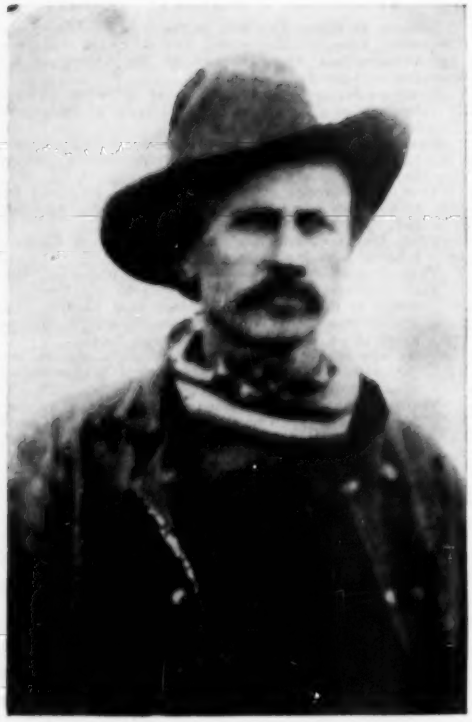

Why the killer came to Powder Springs

THE SKINNY little Texan who drifted into Butch Cassidy's layout at Powder Springs one day in '97 had dead-level eyes, a droopy mustache, and two six-guns tied down for the fast draw. Called himself Carter. Said he was a killer on the run.

That's why Cassidy and the outlaws in his notorious Wild Bunch told him all about the big future plans for their train robbers' syndicate. They took him in.

And he took them in. He was a range detective whose real name was a legend in the West—Charlie Siringo. And the information he got before he quietly slipped away stopped the Wild Bunch for a long, long time.

Of course, Siringo knew all along that if Cassidy or the others had discovered the truth, they'd have killed him sure. But it just never worried him any.

You couldn't scare Charlie Siringo. Coolest of cool customers and rawhide tough, he had the go-it-alone courage it takes to build a peaceable nation out of wild frontier. That brand of courage is part of America and her people—part of the country's strength. And it's a big reason why one of the finest investments you can lay hands on is America's Savings Bonds. Because those Bonds are backed by the independence and courage of 165 million Americans. So buy U. S. Savings Bonds. Buy them confidently—regularly—and hold on to them!

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